Distinct Ankyrin Isoforms at Neuron Cell Bodies and Nodes of Ranvier Resolved Using Erythrocyte Ankyrin–deficient Mice

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Abstract. Isoforms of ankyrin (ankyrins$_R$) immunologically related to erythrocyte ankyrin (ankyrin$_{Ro}$) are associated with distinct neuronal plasma membrane domains of functional importance, such as cell bodies and dendrites, axonal hillock and initial segments, and nodes of Ranvier. Ankyrin$_{Ro}$ is expressed in brain, and accounts for at least one of the ankyrin$_R$ isoforms. Another ankyrin isoform of brain, ankyrin$_B$, is encoded by a distinct gene and is immunologically distinct from ankyrins$_R$. Mutant mice with normoblastosis (*nb/nb*) constitute the first described genetic model of ankyrin deficiency: they display a severe hemolytic anemia due to a significantly reduced expression of the ankyrin$_{Ro}$ gene in reticulocytes as well as brain (Peters L. L., C. S. Birkenmeier, R. T. Bronson, R. A. White, S. E. Lux, E. Otto, V. Bennett, A. Higgins, and J. E. Barker. 1991. *J. Cell Biol.* 114:1233–1241). In the present report, we distinguish between ankyrin$_{Ro}$ and other ankyrin$_R$ isoforms using immunoblot analysis and immunofluorescence localization of ankyrins$_R$ throughout the nervous system (forebrain, cerebellum, brain stem, spinal cord, and sciatic nerve) of *nb/nb* and normal mice. This is the first immunocytochemical characterization of the neurological component of the *nb* mutation and shows the following. (*a*) The isoform of ankyrin at the nodes of Ranvier and initial axonal segments is present in the *nb/nb* mice and does not cross-react with an ankyrin$_{Ro}$-specific antibody; this isoform, therefore, is distinct from both ankyrin isoforms identified in brain, ankyrin$_{Ro}$ and ankyrin$_B$, and is probably the product of a distinct gene and a unique component of the specialized membrane skeleton associated with nodes of Ranvier. (*b*) Ankyrin$_{Ro}$ missing from *nb/nb* mice is selectively associated with neuronal cell bodies and dendrites, excluded from myelinated axons, and displays a selective pattern of expression in the nervous system whereby expression is almost ubiquitous in neurons of the cerebellum (Purkinje and granule cells) and spinal cord, and restricted to a very minor subset of neurons in hippocampus and neocortex of forebrain.

ANKYRIN was initially identified as the principal peripheral membrane protein involved in the attachment of the spectrin-based membrane skeleton to the plasma membrane of human erythrocytes (ankyrin$_{Ro}$) (reviewed in Bennett, 1985, 1990). Ankyrins have subsequently been discovered to be a family of closely related polypeptides associated with the plasma membrane of cells in several tissues, and especially abundant in brain (reviewed in Bennett, 1990). One hypothesized role for ankyrins is in placement of membrane proteins in specialized domains of the plasma membrane. Examples of membrane proteins that associate with ankyrin in in vitro assays and are colocalized in tissues include the voltage-dependent sodium channel (Srinivasan et al., 1988) at nodes of Ranvier (Kordeli et al., 1990) and the neuromuscular junction (Flucher and Daniels, 1989), the Na^+/K^+ ATPase in basal domains of kidney distal tubule cells (Koob et al., 1987; Morrow et al., 1989), and the anion exchanger in basolateral domains of kidney collecting duct cells (Drenckhahn et al., 1985).

Functional diversity between the members of the ankyrin family is generated by the expression of multiple genes as well as alternative splicing of pre-mRNAs. Brain tissue contains at least two types of ankyrin: a major form termed ankyrin$_B$ which is present in most cell types (Kordeli et al., 1990) and comprises 0.5–1% of the total membrane protein (Davis and Bennett, 1984a,b), and a more restricted subfamily termed ankyrin$_R$ detected with antibody against erythrocyte ankyrin (ankyrin$_{Ro}$). Expression of the ankyrin$_{Ro}$ gene itself has been previously shown, by Northern blot analysis, to occur in brain (Lambert et al., 1990), although no information was available regarding the subcellular localization of the encoded polypeptide. Ankyrin$_B$ and ankyrin$_{Ro}$ are encoded by distinct genes that are located on different chromosomes in human (Lambert et al., 1990; Lux et al., 1990b; Otto et al., 1991) and mouse (White et al., 1990; Peters et al., 1991). Therefore, at least two different ankyrin genes are expressed in the nervous system. Alternative mRNA splicing of ankyrin$_{Ro}$ messages has been shown to occur in regions encoding the regulatory domain of the molecule, providing a possible mechanism for the generation of other ankyrin
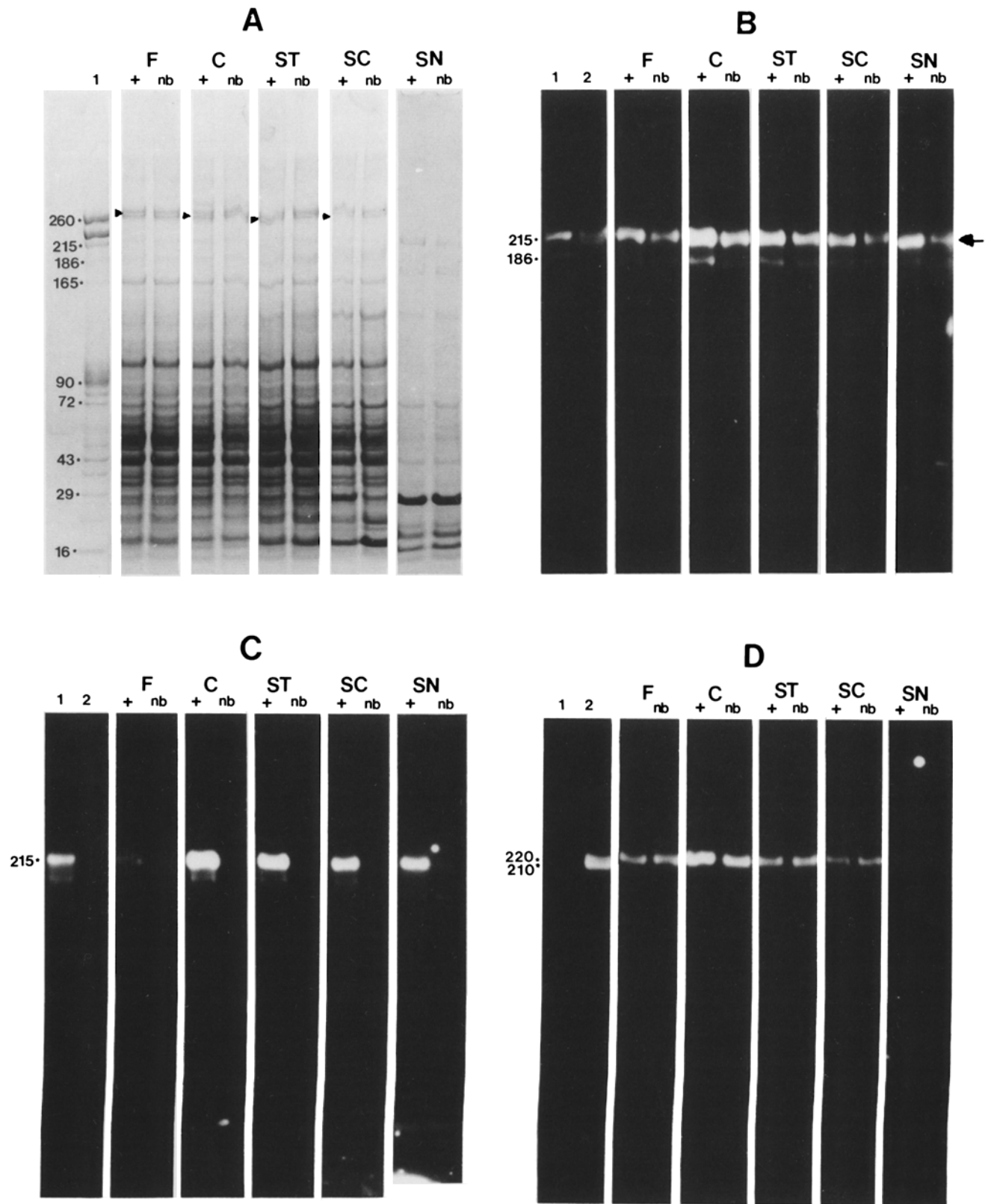
Figure 1. Immunoblot detection of ankyrin isoforms in various regions of the nervous tissue of normal and *nb/nb* mice. Membrane fractions of forebrain (*F*), cerebellum (*C*), brain stem (*ST*), spinal cord (*SC*), and sciatic nerve (*SN*) homogenates from normal (+) and mutant (*nb*) mice were electrophoresed on SDS-PAGE (*A*, Coomassie blue staining), transferred on nitrocellulose paper, and incubated with antibodies raised against RBCank (the total erythrocyte ankyrin polypeptide(s), called ankyrin$_{Ro}$) (*B*), 2.2 Δ, a 17-kD region, unique to the regulatory domain of erythrocyte ankyrin 2.1 polypeptide, that is not present in the 2.2-polypeptide (ankyrin$_{Ro}$-specific antibody) (*C*), and ankyrin$_B$ (the major ankyrin isoform purified from brain tissue) (*D*). Lanes *1* and *2* contain human erythrocyte ghost membranes and purified bovine ankyrin$_B$, respectively. Bovine ankyrin$_B$ migrates as a double band of M_r, 220/210 kD and rat ankyrin$_B$ is a single 220-kD

polypeptides, such as the band 2.2 observed in human erythrocytes (Hall and Bennett, 1987; Lambert et al., 1990; Lux et al., 1990a). Ankyrin$_R$ isoforms of brain are of special interest since these proteins have been localized in neurons in subcellular membrane domains of functional importance, such as: the plasma membrane of neuron cell bodies, where multiple synapses occur (Nelson and Lazarides, 1984; Drenckhahn and Bennett, 1987; Kordeli et al., 1990); axonal hillock and initial segments, where action potential is generated (Kordeli et al., 1990); nodes of Ranvier of myelinated axons, where saltatory conduction of the nerve signal takes place (Kordeli et al., 1990). An unresolved question from these initial studies using antibodies is the degree of diversity within the ankyrin$_R$ family. Alternatives range from the same gene and identical mRNA encoding ankyrin at the node of Ranvier, neuronal cell bodies, and in erythrocytes, to distinct genes and/or alternatively spliced mRNAs for each of these ankyrins.

In this report we distinguish between ankyrin$_{Ro}$ and other ankyrin$_R$ isoforms in the nervous system, using a mutant mouse model (*nb/nb*) that is deficient in ankyrin$_{Ro}$. Normoblastosis (*nb*) is a recessive mutation in a single gene linked to the erythrocyte ankyrin locus Ank-1 on mouse chromosome 8 (White et al., 1990), and results in 90% reduction in expression of ankyrin$_{Ro}$ in erythrocytes with a phenotype of deficiency of spectrin and a severe hemolytic anemia (Bodine et al., 1984; White et al., 1990). This mutation is the first available genetic model of ankyrin deficiency. A surprising recent development has been the finding that the ankyrin$_{Ro}$ mRNA levels are reduced to <10% of normal not only in the reticulocytes, but also in the brains of *nb/nb* mice (Peters, L. L., C. S. Birkenmeier, R. A. White, S. E. Lux, and J. E. Barker. 1989. *Blood*. 74:59a; Peters et al., 1991), involving a neurological component in the normoblastosis mutation. The ankyrin$_{Ro}$ gene in the brain of normal mice is expressed in particularly high levels in the cerebellum, where the mRNA has been localized by in situ hybridization in Purkinje cells and was also missing from the *nb/nb* mice (Peters, L. L., C. S. Birkenmeier, R. Bronson, R. A. White, S. E. Lux, and J. E. Barker. 1990. *Blood*. 76:14a; Peters et al., 1991). The use of the *nb/nb* mice in this study allowed the unambiguous identification of an ankyrin isoform as ankyrin$_{Ro}$ and the secure distinction between ankyrin$_{Ro}$ and other ankyrin$_R$ isoforms in the nervous system.

We established the distribution of a single ankyrin$_R$ isoform, ankyrin$_{Ro}$, throughout the nervous system, and compared that to the localization of the other members of the ankyrin$_R$ family, using immunofluorescence and immunoblot analysis of brain tissue of *nb/nb* and normal mice. As an additional tool, we raised a polyclonal antibody that displays exclusive specificity for ankyrin$_{Ro}$ among other ankyrin isoforms, as confirmed by localization studies involving the mutant mice. The conclusions of this report are that: (a) the isoform of ankyrin that is located at the nodes of Ranvier and initial axonal segments is distinct from both ankyrin isoforms identified in brain, ankyrin$_{Ro}$ and ankyrin$_B$, and is

probably the product of a distinct gene and a unique component of the specialized membrane skeleton associated with nodes of Ranvier, (b) the ankyrin$_{Ro}$ gene product, that is linked to the normoblastosis mutation, is selectively associated with neuronal cell bodies and dendrites, excluded from myelinated axons, and exhibits an unanticipated selectivity in expression in only certain neurons.

Materials and Methods

Animals

Mutant normoblastic (*nb/nb*) mice and normal controls are inbred strains maintained at the Jackson Laboratory (Bar Harbor, Maine), and were kindly provided by Dr. Jane Barker.

Antibodies

Affinity-purified polyclonal antibodies against the 89-kD domain of human erythrocyte ankyrin (Bennett and Stenbuck, 1980) and ankyrin purified from bovine brain (Kordeli et al., 1990) were described previously. The antibody specific for ankyrin$_{Ro}$ (erythrocyte ankyrin) was raised against three synthetic 15-residue peptides from the regulatory domain in an exon that is partially missing from an alternatively spliced form of ankyrin$_{Ro}$ known as protein 2.2 (Lux et al., 1990a; Lambert et al., 1990). The peptides were coupled to glutaraldehyde-activated rabbit serum albumin and the resulting serum was first depleted of reactivity to other regions of ankyrin by passage through a protein 2.2-column, and affinity purified using sheep erythrocyte ankyrin polypeptides coupled to a Sepharose column, in order to obtain antibody capable of reacting with ankyrin of other species.

Procedures

Different parts of nervous tissue (forebrain, cerebellum, brain stem, spinal cord, and sciatic nerve) were obtained from adult (6-7 mo old) WBB6F normal and *nb/nb* mice (Bodine et al., 1984). After decapitation, tissue was rapidly dissected and homogenized with a Polytron (Brinkmann Instruments, Inc., Westbury, NY) in 10 mM sodium phosphate buffer, pH 7.5, 1 mM NaEDTA, 0.32 M sucrose and 1 mM sodium azide. Gel samples were prepared from the postnuclear membrane fraction (1,000 g supernatant, devoid of the majority of erythrocytes) which was pelleted at 100,000 g for 1 h. In the case of the sciatic nerve, due to the small size of the tissue, the 100,000 g pellet of the total homogenate was used to prepare a gel sample. SDS-PAGE and immunoblot analysis were performed as described (Davis and Bennett, 1984a). Erythrocyte ghost membranes (Bennett, 1983) and bovine brain ankyrin (Davis and Bennett, 1984b) were prepared as described.

Immunofluorescence Microscopy

Adult (5 mo old) WBB6F normal and *nb/nb* mice (Bodine et al., 1984) were perfused first with heparin, 1% NaNO$_3$, pH 7.5, followed by 4% paraformaldehyde in 0.1 M Na phosphate, pH 7.5. The spinal cord, forebrain, cerebellum, brain stem, and sciatic nerve were removed and placed in fixative overnight. The tissue was cryoprotected with 0.7 M sucrose, frozen in liquid nitrogen-cooled isopentane, and cut at −20°C with a cryostat. 4-μm-thick sections were mounted on egg white-coated glass slides, air dried, and stored at −80°C. Sections were immunostained using antibodies at 1-5 μg/ml by indirect immunofluorescence (rhodamine-conjugated goat anti-rabbit Ig was from Cappel Laboratories, Malvern, PA). After the immunostaining procedure, the sections were mounted in FITC-Guard (Testog Inc., Chicago, IL) and observed by epifluorescence using a Zeiss 63× objective. As a control, the first antibody was replaced by rabbit nonimmune Ig during incubations.

polypeptide. Arrowheads (*A*) show the two brain spectrin (fodrin) polypeptides. Protein concentration of the corresponding normal and *nb/nb* tissue samples has been normalized by resuspending membrane pellets of equal wet weight in the same volume of buffer. Markers are the molecular masses of erythrocyte membrane proteins including the human erythrocyte ankyrin polypeptides 2.1 (215 kD), 2.2 (186 kD), and 2.3 (165 kD). Exposure of the autoradiogram of ankyrin$_{Ro}$-specific antibody was sevenfold longer than autoradiograms of RBCank and ankyrin$_B$ antibodies. Exposure of the autoradiograms of sciatic nerve (*SN*) was 20-fold longer than the rest of the tissues.
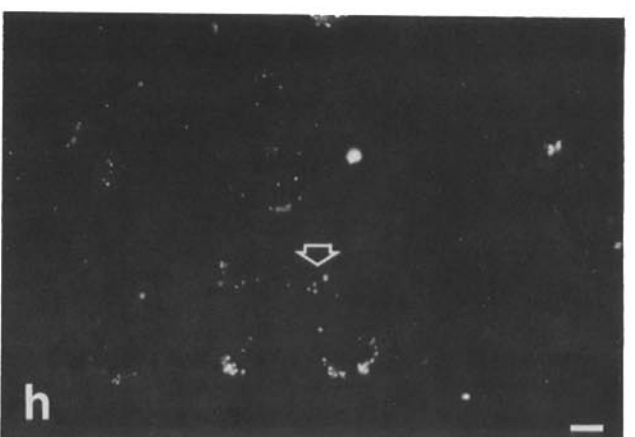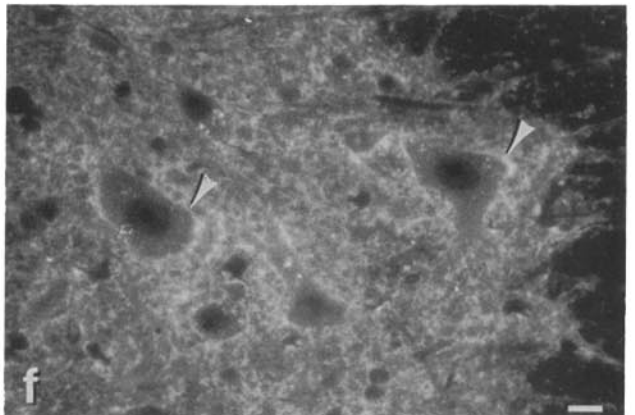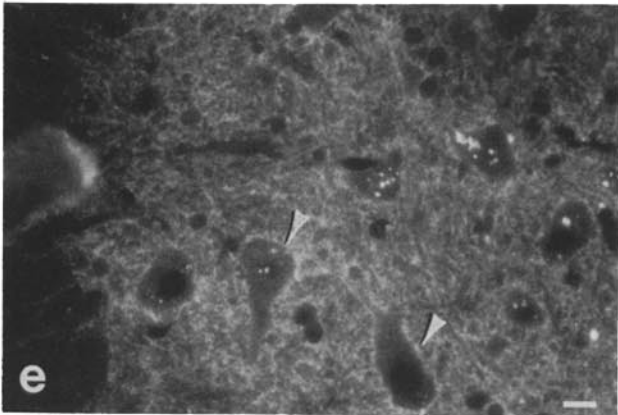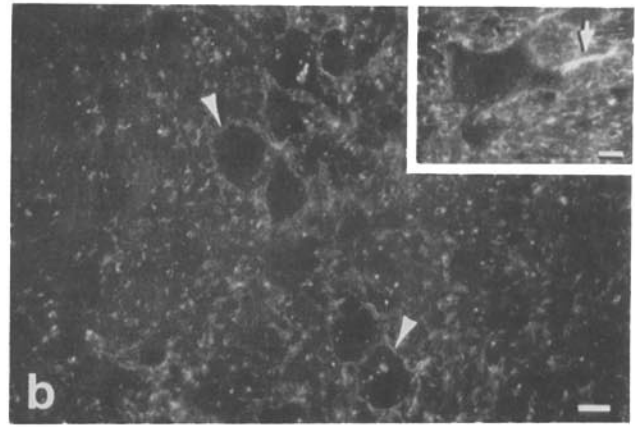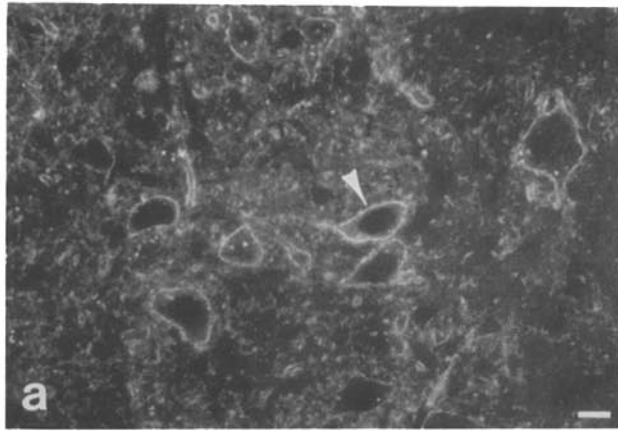
Results

Detection of Ankyrin Isoforms in the Nervous System of Normal and nb/nb Mice

Isoforms of ankyrin have been detected by immunoblots in membrane preparations from different regions of the nervous tissue of nb/nb mice (Fig. 1), using affinity-purified polyclonal antibodies. As a control, homologous tissue samples have been prepared from normal mice of the same strain that do not display any ankyrin or spectrin deficiency.

Antibody directed against the 89-kD domain of human ankyrin$_{Ro}$ (erythrocyte ankyrin) (referred to as RBCank antibody in this paper) (Fig. 1 B) reacts with ankyrin$_{Ro}$ in human erythrocytes (lane 1) and with ankyrin$_{Ro}$-related polypeptides in bovine and rat brain (Davis and Bennett, 1984a,b; Kordeli et al., 1990). This antibody also crossreacts with ankyrin$_B$ (Kordeli et al., 1990), the major form of ankyrin in brain tissue (Fig. 1 B, lane 2, bovine ankyrin$_B$; Kordeli et al., 1990, rat ankyrin$_B$). The cross-reaction between ankyrins is not unexpected since the two ankyrins share 68% sequence identity in the 89-kD domain (Otto et al., 1991). The RBCank antibody crossreacts intensely with two polypeptides (215/186 kD) in the 100,000 g membrane fractions of forebrain, cerebellum, stem, spinal cord, and sciatic nerve of control mice. Brain tissues of nb/nb mice exhibit a slightly (forebrain, stem) or greatly reduced (cerebellum, sciatic nerve, spinal cord) cross-reaction.

An antibody was prepared against a restricted domain of the ankyrin molecule which is highly specific for ankyrin$_{Ro}$. This domain is located at the carboxy terminus of the molecule within the regulatory domain, and is missing from the lower molecular mass form of erythrocyte ankyrin (186 kD, protein 2.2 polypeptide), due to alternative splicing of pre-mRNA within an exon (Lux et al., 1990a; Lambert et al., 1990; Tse, 1990). The ankyrin$_{Ro}$-specific antibody (Fig. 1 C) displays a strong cross-reaction with erythrocyte ghost membranes (lane 1), but not at all with the major form of brain ankyrin (lane 2). In the nervous tissue of control mice, this antibody recognizes a major polypeptide of 215 kD, very similar to the erythrocyte ankyrin polypeptide. The nb/nb mouse exhibits a striking absence of cross-reactivity with this antibody, suggesting that the ankyrin$_{Ro}$ gene product is not present in the membranes of the nervous tissue of nb/nb mice. A trivial explanation for the observed lack of ankyrin$_{Ro}$ in the nb/nb nervous tissue would be contamination of tissue homogenates with erythrocytes: ankyrin$_{Ro}$ of erythrocytes would be present in tissues from normal mice but not from nb/nb mice that (a) are anemic and (b) do not express ankyrin$_{Ro}$ in reticulocytes. Such a possibility has been minimized by the method of preparation of tissue samples (see Materials and Methods), and excluded by immunoblots of these samples with an antibody against the erythrocyte-specific protein anion exchanger (band 3); this antibody recognizes the murine anion exchanger polypeptide (Kordeli

et al., 1990) and revealed minimal contamination of erythrocytes in forebrain, cerebellum, brain stem, and spinal cord (data not shown). However, erythrocyte contamination is a problem in sciatic nerve (see Materials and Methods and below).

No cross-reacting protein was detected with the ankyrin$_{Ro}$-specific antibody in the cytosolic fractions of the same tissue samples of normal and nb/nb mice (not shown). This observation excluded the possibility of the existence of a more labile form of ankyrin$_{Ro}$ that would become destabilized and released in the cytosol during the preparation of the membrane fractions.

In one immunoblot experiment, using much more concentrated samples and long exposure of autoradiograms, a very small amount of ankyrin polypeptides has been observed in the nb/nb tissues (data not shown). The pattern of those polypeptides was identical to the normal tissues, and probably results from residual expression of erythrocyte ankyrin in nb/nb mice (Peters, L. L. 1990. Blood. 76:14a; Peters et al., 1991). Exposure of the autoradiograms from the ankyrin$_{Ro}$-specific antibody was seven times longer than in the case of RBCank antibody, suggesting that ankyrin$_{Ro}$ is a relatively minor isoform in most parts of the nervous system and especially in the case of the forebrain (Fig. 1 C, F+). Low abundance of the ankyrin$_{Ro}$ isoform is confirmed by immunocytochemistry (see below).

Minor, lower molecular mass bands are also detected in autoradiograms (but are not reproduced well in published prints) in both the erythrocyte ghosts and the nervous tissue by the ankyrin$_{Ro}$-specific antibody (Fig. 1 C). These are ankyrin$_{Ro}$-related polypeptides, since: (a) incubation of the ankyrin$_{Ro}$-specific antibody with an excess of purified, 215-kD erythrocyte ankyrin, before incubation with normal nervous tissue sections, completely inhibits labeling (not shown); (b) these polypeptides are also missing from the mutant nervous tissue homogenates. Despite a similar molecular mass (186 kD), these polypeptides are most probably distinct from the alternatively spliced 186-kD form of ankyrin in erythrocytes (band 2.2; Hall and Bennett, 1987; Lux et al., 1990a; Lambert et al., 1990) since the erythrocyte form lacks the sequence used to prepare this particular antibody. The 186-kD cross-reactive bands, therefore, may represent another product of alternative splicing of ankyrin mRNA or result from proteolysis with calpain (Hall and Bennett, 1987). It is noteworthy that at least one other site is alternatively spliced at the carboxyl terminus of erythrocyte ankyrin (Lambert et al., 1990).

Antibody against the major form of ankyrin purified from bovine brain (Fig. 1 D) detects equal amounts of this isoform (ankyrin$_B$) in the nervous tissue of both control and nb/nb mice, indicating that this isoform is normally expressed in the mutant mice. Sciatic nerve did not cross react with this antibody, suggesting that peripheral nerve may contain another isoform of ankyrin.

Immunoblot data obtained with the RBCank antibody

Figure 2. Immunofluorescence localization of ankyrin isoforms on cryosections of normal and nb/nb spinal cord. Cross cryosections of spinal cord grey matter from normal and nb/nb mice have been labeled with the RBCank antibody (a and b, respectively), the ankyrin$_{Ro}$-specific antibody (c and d, respectively), and the ankyrin$_B$ antibody (e and f, respectively). Inset in b shows the labeled initial axonal segment (arrow) of a motor neuron cell. In control experiments, the first antibody was replaced by rabbit nonimmune Ig during incubations (g and h for normal and nb/nb tissue, respectively). Arrowheads point to plasma membranes of neuron cells and open arrows to autofluorescing lipofuskin granules within the neuron cell cytoplasm. Bars, 10 μm.
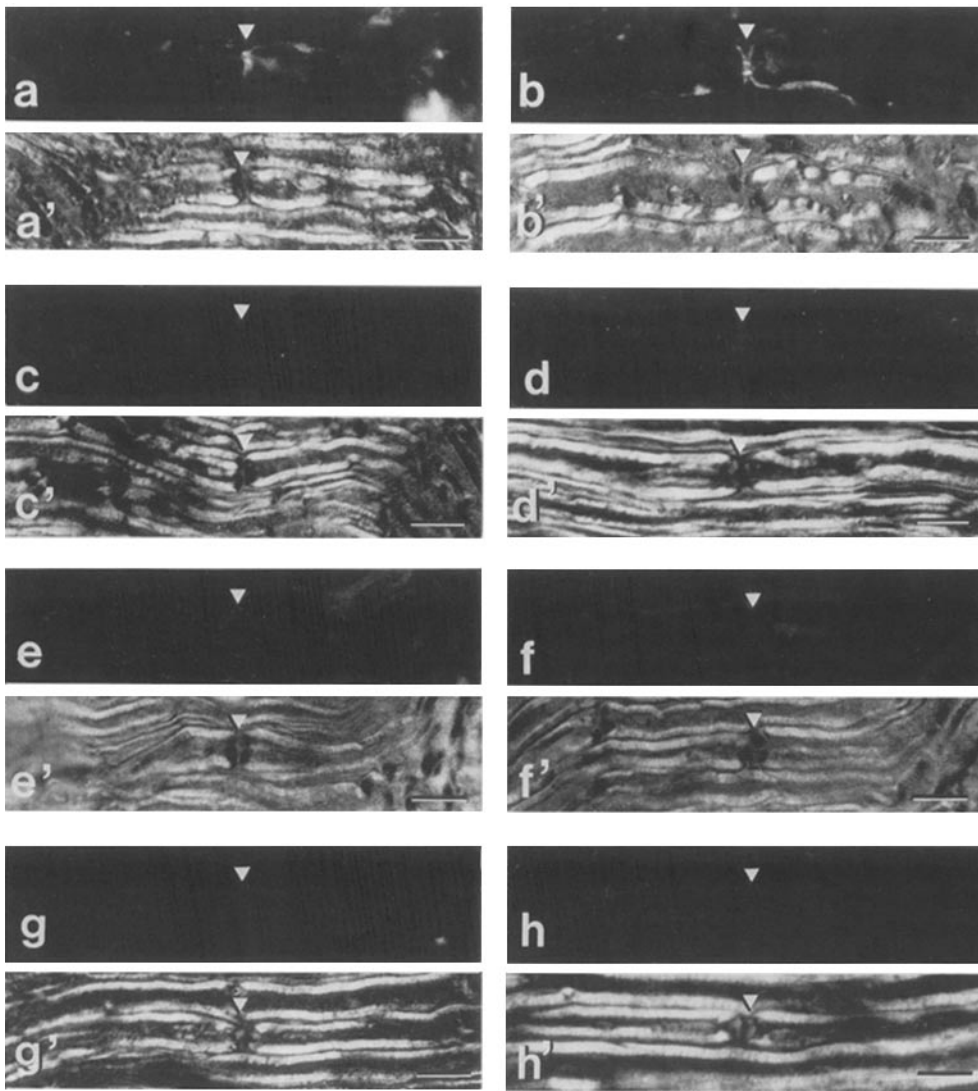
Figure 3. Immunofluorescence localization of ankyrin isoforms on cryosections of normal and *nb/nb* sciatic nerve. Longitudinal cryosections of sciatic nerve from normal and *nb/nb* mice have been labeled with the RBCank antibody (*a* and *b*, respectively), the ankyrin$_{Ro}$-specific antibody (*c* and *d*, respectively) and the ankyrin$_B$ antibody (*e* and *f*, respectively). In control experiments, the first antibody was replaced by rabbit non-immune Ig during incubations (*g* and *h* for normal and *nb/nb* tissue, respectively). Arrowheads point to nodes of Ranvier. *a'–h'* are DIC micrographs corresponding to immunofluorescence micrographs *a–h*. Bars, 10 μm.

strongly suggest that ankyrin isoforms in addition to ankyrin$_B$ are expressed in the nervous system of *nb/nb* mice. The polypeptides that can still be detected in the mutant mice with this antibody cannot correspond to the ankyrin$_{Ro}$ gene product; they rather seem to be different ankyrin isoforms immunologically related to the ankyrin$_{Ro}$ gene product, as judged by cross-reactivity with the antibody that recognizes ankyrin in erythrocyte membranes, but not with the ankyrin$_B$-specific antibody.

The Coomassie blue–stained SDS-PAGE gel of normal and *nb/nb* nervous tissue samples (Fig. 1 *A*) shows that several polypeptides other than ankyrin appear to differ in relative quantities between *nb/nb* mice and normals. For instance, two polypeptides of M_r 23 and 29 kD appeared reduced in mutant cerebellum homogenates from two different membrane preparations. Brain spectrin (fodrin) polypeptides, as detected in Coomassie blue–stained gels (Fig. 1 *A*, *arrowheads*), are present in unaltered amounts in *nb/nb* brain, in contrast to *nb/nb* erythrocytes where spectrin is reduced by 50% (Bodine et al., 1984).

Immunofluorescence Localization of Ankyrin Isoforms in the Spinal Cord and the Peripheral Nerve of Normal and nb/nb Mice

Selective absence of ankyrin$_{Ro}$ from the membrane fractions of nervous tissue of the *nb/nb* mice raised the important question of the localization of that particular isoform in normal nervous tissue.

Labeling of 4-μm-thick, frozen cross sections of spinal cord from normal mice with the RBCank antibody (Fig. 2 *a*) was concentrated around neuron cells of the gray matter in a punctate pattern (*arrowhead*), while the ankyrin$_B$ antibody generated a general labeling of the neuropil of the gray matter, and a rather diffuse labeling around the neurons (Fig. 2 *e*). The ankyrin$_{Ro}$-specific antibody labeled selectively the neuron cell bodies and starting parts of dendrites in the grey matter (Fig. 2 *c*). The punctate labeling around cell bodies is suggestive of a localization in the postsynaptic areas; nevertheless, at this level of resolution, localization at the presynaptic areas as well, or even at the plasma membrane domains between synapses, cannot be excluded. In the *nb/nb*

mice, neuronal cell body labeling was very much reduced or completely absent when using the RBCank and the ankyrin$_{Ro}$-specific antibodies, respectively (Fig. 2, b and d, respectively). Interestingly, intensely labeled axonal initial segments could still be observed in the gray matter of nb/nb mice (Fig. 2 b, inset). Labeling of the ankyrin$_B$ antibody did not show any difference between normal and mutant mice (Fig. 2, e and f, respectively).

In longitudinal sections of sciatic nerve of normal mice, the RBCank antibody (Fig. 3 a, arrowhead) intensely labeled nodes of Ranvier, as previously described in rat (Kordeli et al., 1990). Interestingly, labeling of the nodes of Ranvier was identical in the sciatic nerve of normal and nb/nb mice (Fig. 3 b), indicating that the ankyrin isoform located at the nodes of Ranvier is distinct from ankyrin$_{Ro}$. Absence of labeling of the nodes of Ranvier of both control and nb/nb mice with the ankyrin$_{Ro}$-specific antibody provides further evidence for a specialized form of ankyrin at the nodes of Ranvier (Fig. 3, c and d, respectively). Antibody against ankyrin$_B$ (Fig. 3, e and f) did not cross react with the nodes of Ranvier either, suggesting that the ankyrin isoform at the nodes of Ranvier is a member of the ankyrin$_R$ family. Some axoplasmic labeling has been observed with the ankyrin$_{Ro}$-specific antibody and especially with the ankyrin$_B$ antibody. Axoplasmic labeling persisted in the nb/nb sciatic nerve, varied between individual experiments, and could be due to a cross-reaction of the antibodies with a common epitope shared between ankyrin and another protein(s), as observed for the cytoplasmic and nuclear labeling of the neuron cells with the ankyrin$_{Ro}$-specific antibody (see below).

The 215-kD polypeptide detected with RBCank antibody in sciatic nerve of nb/nb mice (Fig. 1 B, arrow) is likely to represent the form of ankyrin at the nodes of Ranvier and/or unmyelinated axons (Kordeli et al., 1990). This band is not explained by contaminating erythrocytes since nb/nb mice almost entirely lack erythrocyte ankyrin and in addition are anemic. However, the 215-kD band detected with both RBCank and ankyrin$_{Ro}$-specific antibodies in normal sciatic nerve (Fig. 1, B and C, SN+) most likely does represent a substantial (at least 80%) contribution from contaminating erythrocyte membranes (see Materials and Methods), as confirmed by cross-reaction of these samples on parallel blots with an antibody against the erythrocyte-specific protein anion exchanger (band 3) (not shown).

Immunofluorescence Localization of Ankyrin Isoforms in Different Regions of the Brain of Normal and nb/nb Mice

Frozen 4-μm-thick sections were prepared from three major regions of the brain of normal and nb/nb mice: the cerebellum, the brain stem and the remaining part of the forebrain.

Localization of ankyrin in cross sections of cerebellum of normal mice using the RBCank antibody (Fig. 4 a) revealed a strong, general labeling of the molecular layer that consists mainly of Purkinje cell dendrites, the parallel unmyelinated fibers of the granule neurons, and their synapses. Label also occurred around the Purkinje cells and the granule neurons (granular layer). The punctate labeling around the Purkinje cell bodies suggests labeling of the plasma membrane. Within the granular layer, labeling seems to occur between the granule neurons (Fig. 4 a, arrow), where synaptic com-

plexes (glomeruli) reside, although it is not excluded that plasma membranes of the granule cells are also labeled. A dramatic reduction or absence of labeling around the Purkinje cells and within the granular layer was observed in the cerebellum of nb/nb mice (Fig. 4 b), although the general labeling pattern of the molecular layer appeared quite similar to the normal tissue with this antibody.

As expected from the immunoblot experiments, the difference between control and nb/nb mice became striking when the ankyrin$_{Ro}$-specific antibody was used in immunofluorescence in the cerebellum. Ankyrin$_{Ro}$ is associated with the plasma membranes of Purkinje cells (Fig. 4 c, arrowheads) as well as the molecular and granular layers. In the molecular layer, labeling appeared punctate and could occur on the unmyelinated parallel axons and/or the Purkinje cell dendrites. Although localization at a higher resolution is needed to unambiguously identify the labeled structures of the molecular layer, this labeling occurred on tubular and ring-shaped structures that are reminiscent of the numerous cross-sectioned spines along the proximal and distal Purkinje cell dendrites. Labeling of the granular layer was identical to the RBCank antibody labeling. Labeling was completely lacking from the nb/nb mice (Fig. 4 d), except for a diffuse fluorescence present within the cytoplasm of the neuron cells and dendrites, as well as in the nuclei. The nuclear and cytoplasmic labeling persists in the nb/nb mice, suggesting reaction with an epitope shared between ankyrin and another protein rather than ankyrin$_{Ro}$ itself.

Antibody against ankyrin$_B$ (Fig. 4 e) in normal tissue revealed a general labeling of the molecular layer. Labeling was weak in the granular layer and was not prevalent around the Purkinje cells. As expected by the immunoblot results, no difference in the labeling between cerebellum of nb/nb and control mice (Fig. 4, f and e, respectively) was observed.

Two regions of the forebrain were observed by immunofluorescence: the hippocampus (Fig. 5) and the cerebral cortex (neocortex), opposite to the hippocampal structure (Fig. 6). In the hippocampal structure of the normal mice, RBCank antibody intensely labeled the layer of the pyramidal cells (p) in Ammon's horn, as well as the layer of the granule cells (g) of the dentate gyrus and especially their mossy fibers (asterisk) (Fig. 5, a and a'). Labeling around the neuron cells appeared punctate. This labeling was essentially the same in the nb/nb tissue (Fig. 5, b and b'). Interestingly, the ankyrin$_{Ro}$-specific antibody displayed a very selective, punctate labeling of only a few neuron cells in the pyramidal layer (Fig. 5, c and c', arrowheads), while the granule cell layer completely lacked cell membrane labeling. No labeling at all was observed around neuron cells of both pyramidal and granule cells in the nb/nb tissue with the ankyrin$_{Ro}$-specific antibody (Fig. 5, d and d'). Labeling of nuclei (Fig. 5, d and d', small arrows) was present in both normal and nb/nb tissues, and was not due to ankyrin$_{Ro}$. Ankyrin$_B$ antibody also labeled mossy fibers (asterisk) in both normal (Fig. 5, e and e') and nb/nb tissue (Fig. 5, f and f'), although labeling around pyramidal and granule cells was weak.

In the different layers of the neocortex of normal mice, the neuropil between the pyramidal and nonpyramidal cells was labeled by both RBCank and ankyrin$_B$ antibodies (Fig. 6, a and e, respectively). In addition, labeling of selected neurons (Fig. 6 a, arrowheads) could be observed with the RBCank antibody in normal cortex. The general labeling pattern with
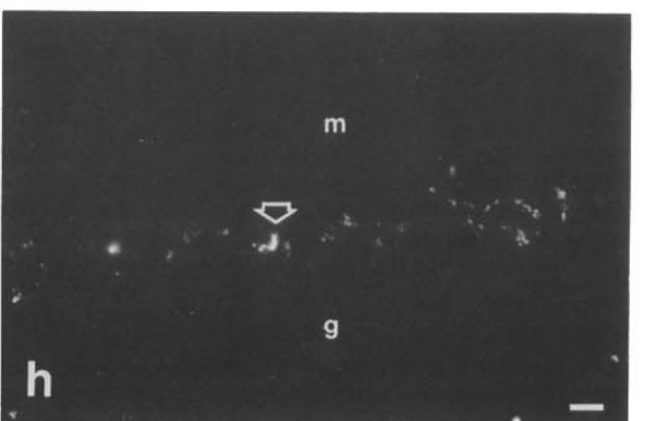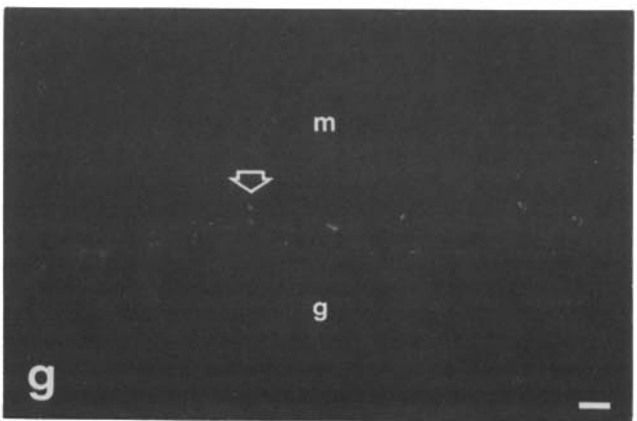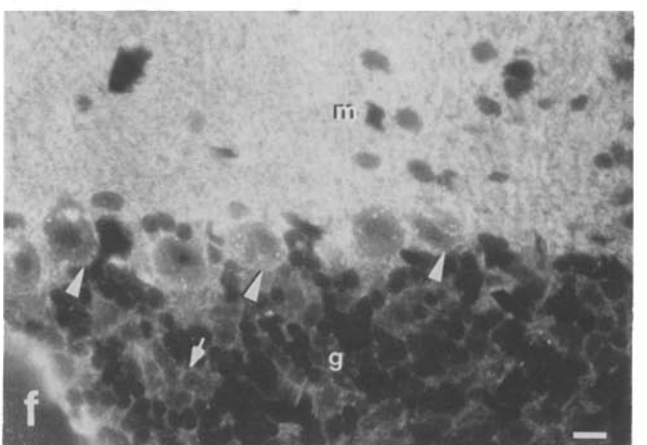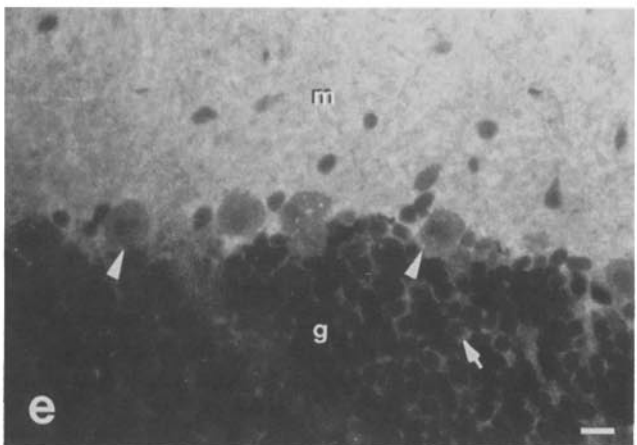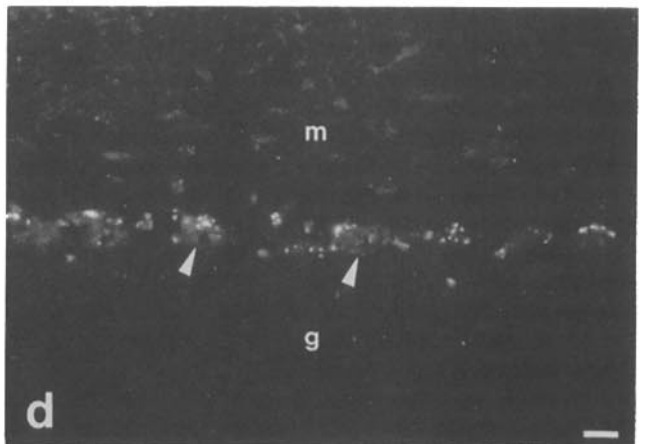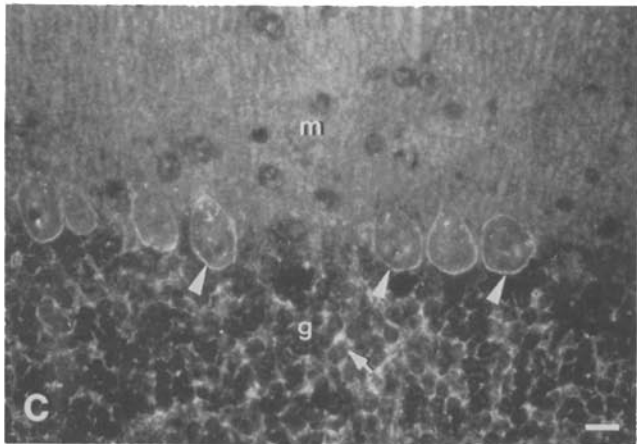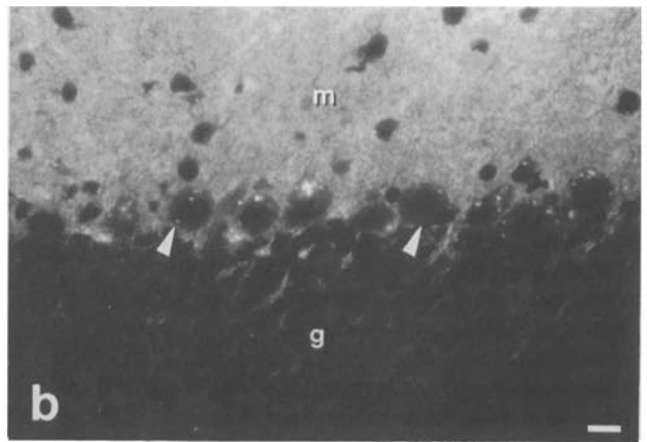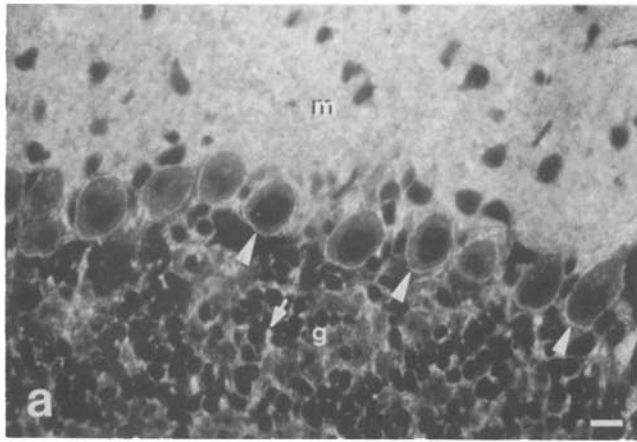
the two antibodies did not change in the *nb/nb* mice (Fig. 6, *b* and *f*) with the exception that selected labeling of neuron cells with RBCank antibody (Fig. 6 *b*) was very much reduced.

The ankyrin$_{Ro}$-specific antibody displayed an impressive selectivity within cortex by labeling only a minor subset of neuron cells in the control mice (Fig. 6 *c, large arrowheads*). No labeling in structures other than these neurons was observed with the ankyrin$_{Ro}$-specific antibody, except for labeling of nuclei, observed in other areas of brain as well. Selective labeling with the ankyrin$_{Ro}$-specific antibody was completely missing from the cortex of the *nb/nb* mice, where only labeled nuclei could be observed (Fig. 6 *d, small arrowheads*). Labeling of selected neurons with both the RBCank and the ankyrin$_{Ro}$-specific antibodies supports the idea that most antigens interacting with the RBCank antibody are related to erythrocyte ankyrin, although it is not obvious whether the same cells are labeled in both cases.

Brain stem appears similar to the other parts of brain regarding the ankyrin$_{Ro}$ localization. Plasma membranes of neuron cell bodies and starting parts of dendrites also appear labeled with both RBCank (Fig. 7 *a*) and ankyrin$_{Ro}$-specific antibodies (Fig. 7 *c*); such labeling is very much reduced or absent in similar areas of *nb/nb* tissue (Fig. 7, *b* and *d*, respectively). Labeling of plasma membranes of neuron cells with ankyrin$_{Ro}$-specific antibody in *nb/nb* tissue was absent from all areas of the sections observed. It is also interesting that in another area of the same section of normal tissue, a class of neuron cells displayed no labeling with RBCank or ankyrin$_{Ro}$-specific antibodies (Fig. 7, *a* and *c*, *insets*). No difference between normal and *nb/nb* tissue has been observed when the ankyrin$_B$ antibody was used (Fig. 7, *e* and *f*, respectively). This antibody also labeled the bundles of CNS myelinated axons, as previously observed (Fig. 7, *e* and *f, asterisks*; Kordeli et al., 1990).

Discussion

Ankyrin$_R$ subtypes in brain were distinguished in this report using a mutant normoblastic mouse (*nb/nb*) with >90% reduction in expression of the erythrocyte ankyrin (ankyrin$_{Ro}$) polypeptide, as well as antibody selective for a single exon of ankyrin$_{Ro}$. Ankyrin$_{Ro}$ is expressed in neuron cells in addition to the major form of ankyrin in brain, ankyrin$_B$, and is missing in normoblastic (*nb/nb*) mice. This form of ankyrin is localized at the plasma membrane around the cell body and dendrites in a punctate pattern, is present in synaptic complexes (glomeruli) between granule neurons of cerebellum, and is not observed in myelinated and recognizable unmyelinated (e.g., hippocampal mossy fibers) axons. Ankyrin$_{Ro}$, therefore, could be selectively associated with postsynaptic domains of somatic and dendritic synapses, although this proposal should be confirmed by high resolution localization studies. Another isoform of ankyrin$_R$ is present in

axonal initial segments and the axolemma of nodes of Ranvier in myelinated axons. This form of ankyrin is retained in *nb/nb* mice and does not cross react with antibody selective for ankyrin$_{Ro}$. Ankyrin at the nodes of Ranvier and initial segments thus is distinct from ankyrin$_{Ro}$ and may represent the product(s) of a distinct gene. The second point of this report is that ankyrin$_{Ro}$ exhibits a highly selective expression in certain neurons throughout the nervous system. In spinal cord most of the neuron cells are labeled, and in the cerebellum both major types of neurons, the Purkinje and granule cells, contain ankyrin$_{Ro}$. However, in the forebrain a very minor subset of neurons were labeled with antibody selective for ankyrin$_{Ro}$.

The *nb/nb* mouse model demonstrates that defects or deficiencies of members of the ankyrin family are likely to be of clinical importance. The prototypic example of a disease involving ankyrin is dominant hereditary spherocytosis (HS), which is a hereditary hemolytic anemia that has been mapped in some families to the short arm of human chromosome 8 (gene symbol SPH1). SPH1 is associated with a deficiency of ankyrin in some cases and cosegregates with ankyrin based on restriction length fragment polymorphism analysis of one kindred (Costa et al., 1990). Biochemical and genetic data suggest that both the SPH1 mutation in man and the *nb* mutation in mouse are caused by lesions in the ankyrin$_{Ro}$ gene which is located on chromosome 8 (Lux et al., 1990*b*; White et al., 1990; reviewed in Palek and Lambert, 1990). The *nb/nb* mice exhibit neurological deficits in addition to a severe anemia. These mice have a tremor, suffer from ataxia, and sustain a progressive degeneration of Purkinje cells (Peters, L. L., C. S. Birkenmeier, R. Bronson, R. A. White, S. E. Lux, and J. E. Barker. 1990. *Blood*. 76: 14a; Peters et al., 1991). The role of ankyrin deficiency in cell death remains to be studied. One possibility is that cell degeneration is due to the gradual disintegration of the peripheral membrane skeleton owing to absence of the linking ankyrin$_{Ro}$ molecule, causing subsequent destabilization of specialized membrane domains such as postsynaptic areas. Another hypothesis would be that absence of ankyrin causes downregulation of an ankyrin-associated membrane protein which is important for the neuron cell survival (e.g., a growth factor receptor).

Previous studies have localized ankyrin$_R$ isoform(s) to several subcellular neuronal membrane sites of functional importance, suggesting a role for ankyrin$_R$ in the organization of the multiple differentiated membrane domains of nervous tissue. Such a role would require a certain degree of specificity in interactions of ankyrin$_R$ with plasma membrane as well as spectrin. However, ankyrin$_R$ immunoreactivity was localized at membrane sites with different function and molecular composition, such as neuronal cell bodies and dendrites (domains rich in neurotransmitter receptors), and initial axonal segments and nodes of Ranvier (domains characterized by a high concentration of the voltage-dependent sodium channel and Na$^+$/K$^+$ ATPase). Further-

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Figure 4. Immunofluorescence localization of ankyrin isoforms on cryosections of normal and *nb/nb* cerebellum. Cryosections of cerebellum from normal and *nb/nb* mice have been labeled with the RBCank antibody (*a* and *b*, respectively), the ankyrin$_{Ro}$-specific antibody (*c* and *d*, respectively) and the ankyrin$_B$ antibody (*e* and *f*, respectively). In control experiments, the first antibody was replaced by rabbit nonimmune Ig during incubations (*g* and *h* for normal and *nb/nb* mice, respectively). Arrowheads point to Purkinje cell plasma membrane, arrows to granule cells and open arrows to autofluorescing lipofuskin granules within the neuron cell cytoplasm. *m*, molecular layer; *g*, granular layer. Bars, 10 μm.
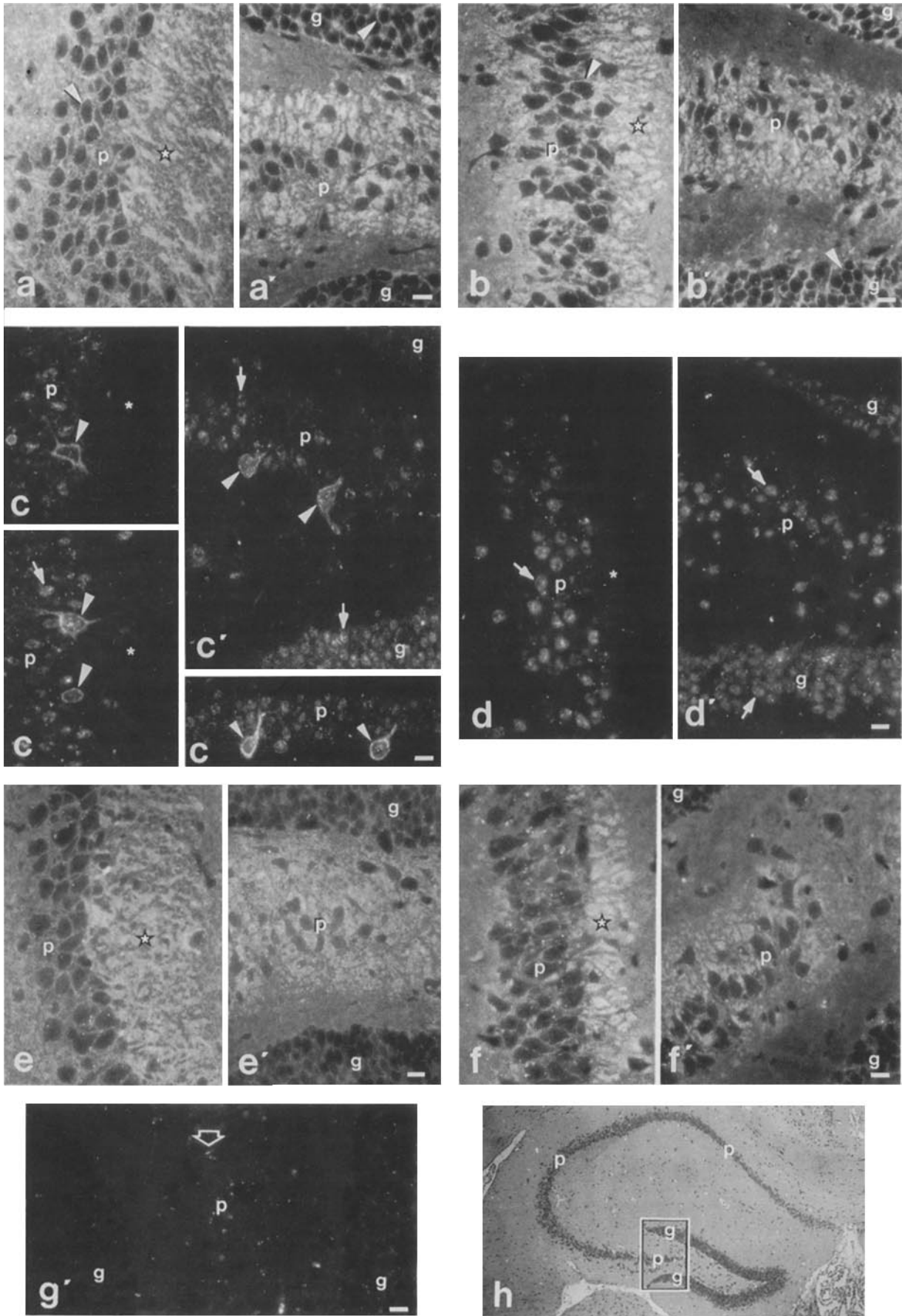
more, ankyrin$_{Ro}$ binds with high affinity to erythrocyte spectrin, but only poorly to brain spectrin (fodrin) (Bennett et al., 1982; Howe et al., 1985). Yet, erythrocyte spectrin is restricted to neuronal cell bodies and dendrites, whereas only brain spectrin appears to undergo axonal transport (Lazarides et al., 1984), and is expected to interact with ankyrin$_R$ at nodes of Ranvier. The answer to these apparent discrepancies would rely upon the degree of diversity within the ankyrin$_R$ family. In this report, we provide the first solid evidence for such a diversity.

An attractive hypothesis regarding the existence of ankyrin isoforms exclusively associated with the plasma membrane of the neuron cell bodies is that different ankyrin isoforms contribute to the accumulation of different integral proteins to particular membrane domains (e.g., segregation of a class of ion channels at a particular type of synapse). This function can be served by specific interactions between ankyrin isoforms and families of integral proteins (Davis et al., 1989) and subsequent linkage to the underlying spectrin-based skeleton. Different types of neurotransmitter receptors as well as different isoforms of particular receptors are known to coexist within the same neuronal subsets (Schofield et al., 1990). Interesting examples of differential subcellular localization of distinct isoforms of ion channels within the same neuron cell include the voltage-dependent sodium channel (Westenbroek et al., 1989) and the muscarinic receptor (Buckley et al., 1988).

A very interesting observation was that, although distribution of the ankyrin$_{Ro}$ was almost ubiquitous in neuron cells of the cerebellum (Purkinje and granule cells) and the spinal cord, only few, selected cells were labeled in the cerebral cortex and the hippocampus. Similarly, in brain stem not all the observed neuron cells were labeled with this antibody. The cell-specific distribution emphasizes the hypothesis that ankyrin$_{Ro}$ may participate in the mechanism(s) generating specific membrane domains in neurons. It is of interest that dystrophin exhibits a distribution similar to that of ankyrin$_{Ro}$: dystrophin labeling occurs in a punctate pattern around cell bodies and dendrites of Purkinje cells, and of only a subset of cortical neurons, and it is not found in axons (Lidov et al., 1990). In addition, the authors demonstrated that punctate labeling, in this case, corresponded to postsynaptic domains. Dystrophin, therefore, being a member of the spectrin family, has been suggested to anchor receptors, or other elements of the postsynaptic apparatus, at appropriate locations of neuronal membranes.

The identification of the selected cortical and hippocampal neurons that contain ankyrin$_{Ro}$ will be a very informative step towards understanding the nature of selectivity in the expression of this ankyrin isoform within neurons. On the other hand, restricted expression of ankyrin$_{Ro}$ suggests that this polypeptide is not the only isoform of ankyrin that is localized at the plasma membrane of the neuron cell bodies. Labeling of the neurons of the nb/nb mice was clearly reduced but not completely absent when using an antibody crossreacting with a highly conserved domain that is a common feature of the ankyrin family. These results suggest again that more than one ankyrin isoform(s) are associated with, and in some occasions coexist at, the cell bodies of neurons.

A major issue of the present study is that a distinct ankyrin$_R$ isoform could be a unique component of the differentiated cortical skeleton that is associated with the nodes of Ranvier. The node of Ranvier is a highly specialized membrane domain of the myelinated axon, characterized by an accumulation of several integral membrane proteins such as ion channels, voltage-dependent sodium channels (reviewed in Waxman and Ritchie, 1985), and the Na$^+$/K$^+$ ATPase (Ariyasu et al., 1985)–cell adhesion glycoproteins such as L1 (Mirsky et al., 1986), N-CAM, Ng-CAM (Rieger et al., 1986), and the extracellular matrix protein cytotactin (Rieger et al., 1986). This membrane domain is associated with a specialized membrane skeleton, the subaxolemmal densities, that are believed to participate in the formation and/or maintenance of the node of Ranvier (Wiley-Livingston and Ellisman, 1980). Little is known about the molecular composition of this skeleton. F-Actin as well as fodrin have been shown to accumulate in these densities, although these proteins are not unique to the nodal membrane domain (Koenig and Repasky, 1985; Zimmerman and Vogt, 1989). An ankyrin$_R$ isoform has been shown recently to be localized on the cytoplasmic surface of the axonal plasma membrane at nodes of Ranvier (Kordeli et al., 1990), using an antibody that was raised against ankyrin$_{Ro}$ and cross reacts with several ankyrin isoforms (RBCank antibody). Ankyrin associates in vitro with the voltage-dependent sodium channels and the Na$^+$/K$^+$ ATPase (Srinivasan et al., 1988; Nelson and Veshnock, 1987). Therefore, this particular ankyrin isoform is a good candidate to participate in segregation of the integral membrane proteins at the nodes of Ranvier. This isoform is at least immunologically related to ankyrin$_{Ro}$, and is not detected by ankyrin$_B$-specific antibody (Kordeli et al., 1990). Interestingly, the labeling of the nodes of Ranvier remained unaffected in the myelinated axons of the nb/nb mice, and the ankyrin$_{Ro}$-specific antibody does not display any cross-reaction with the nodes of Ranvier. These results demonstrate that ankyrin at the node of Ranvier is clearly distinct from ankyrin$_{Ro}$, and presumably is another member of the ankyrin$_R$ family. These findings also suggest that ankyrin at the node of Ranvier is encoded by a gene distinct from ankyrin$_B$ and ankyrin$_{Ro}$ genes. However, the possibility that it is the product of an alternatively spliced ankyrin$_{Ro}$
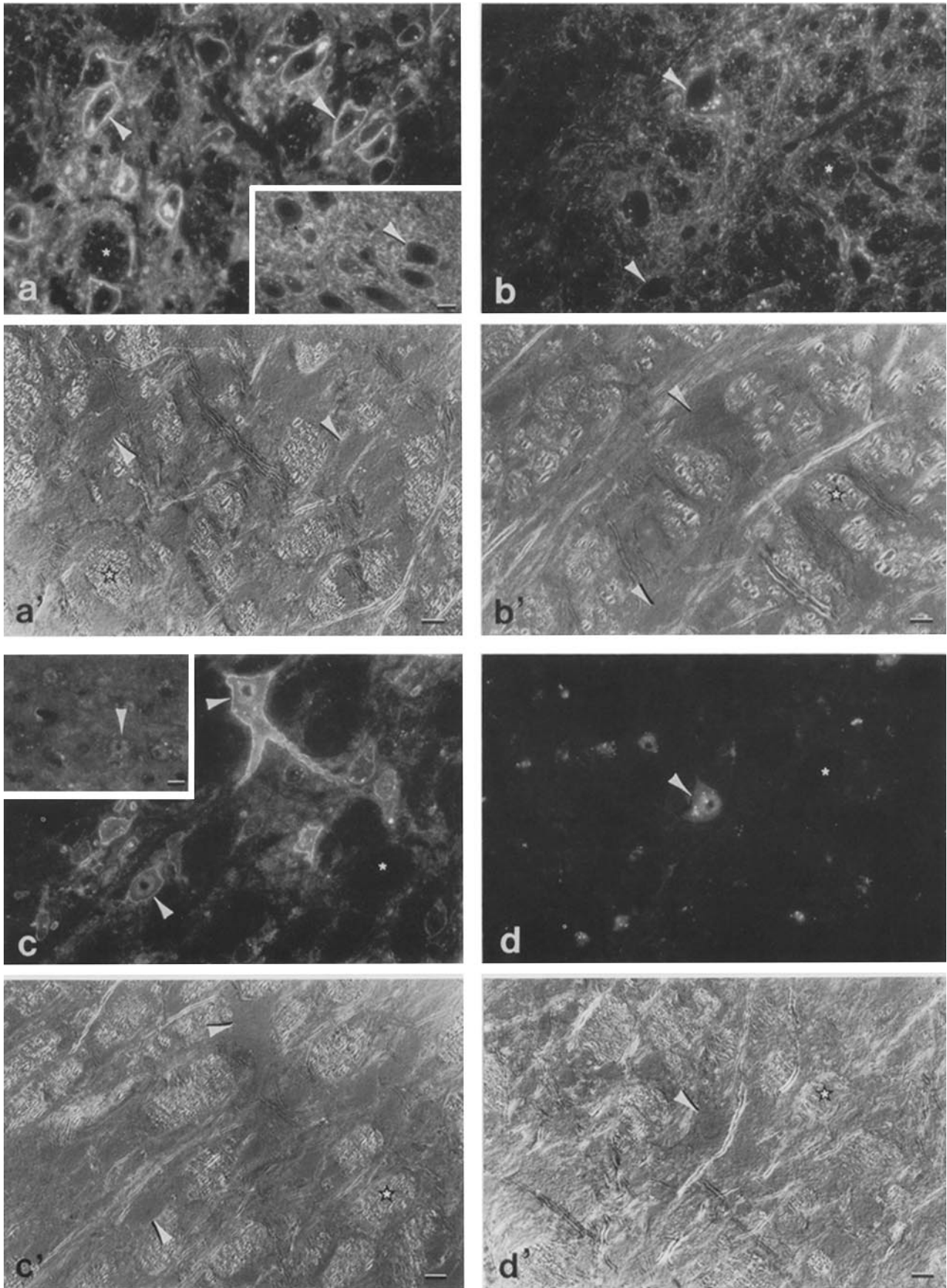
Figure 5. Immunofluorescence localization of ankyrin isoforms on cryosections of normal and nb/nb hippocampus. Coronal cryosections of hippocampal structure from normal and nb/nb mice have been labeled with the RBCank antibody (*a, a'* and *b, b',* respectively), the ankyrin$_{Ro}$-specific antibody (*c, c'* and *d, d',* respectively), and the ankyrin$_B$ antibody (*e, e'* and *f, f',* respectively). *h* is a histologically stained coronal cryosection of normal hippocampus and the underlined area is homologous to the areas shown in *a'–g'. a–f* are from various areas of the pyramidal layer (CA3-CA1) of hippocampus. In control experiments, the first antibody was replaced by rabbit nonimmune Ig during incubations (*g,* normal tissue). Control experiment of nb/nb tissue was identical to the normal tissue (not shown). *p,* layer of pyramidal cells in Ammon's horn; *g,* layer of granule cells in dentate gyrus. Arrowheads point to labeled plasma membranes of neuron cells in both pyramidal and granular layers, short arrows to nuclei, open arrows to autofluorescing lipofuskin granules within the neuron cell cytoplasm, and asterisks indicate the mossy fibers of granule cells. Bars, 10 μm.

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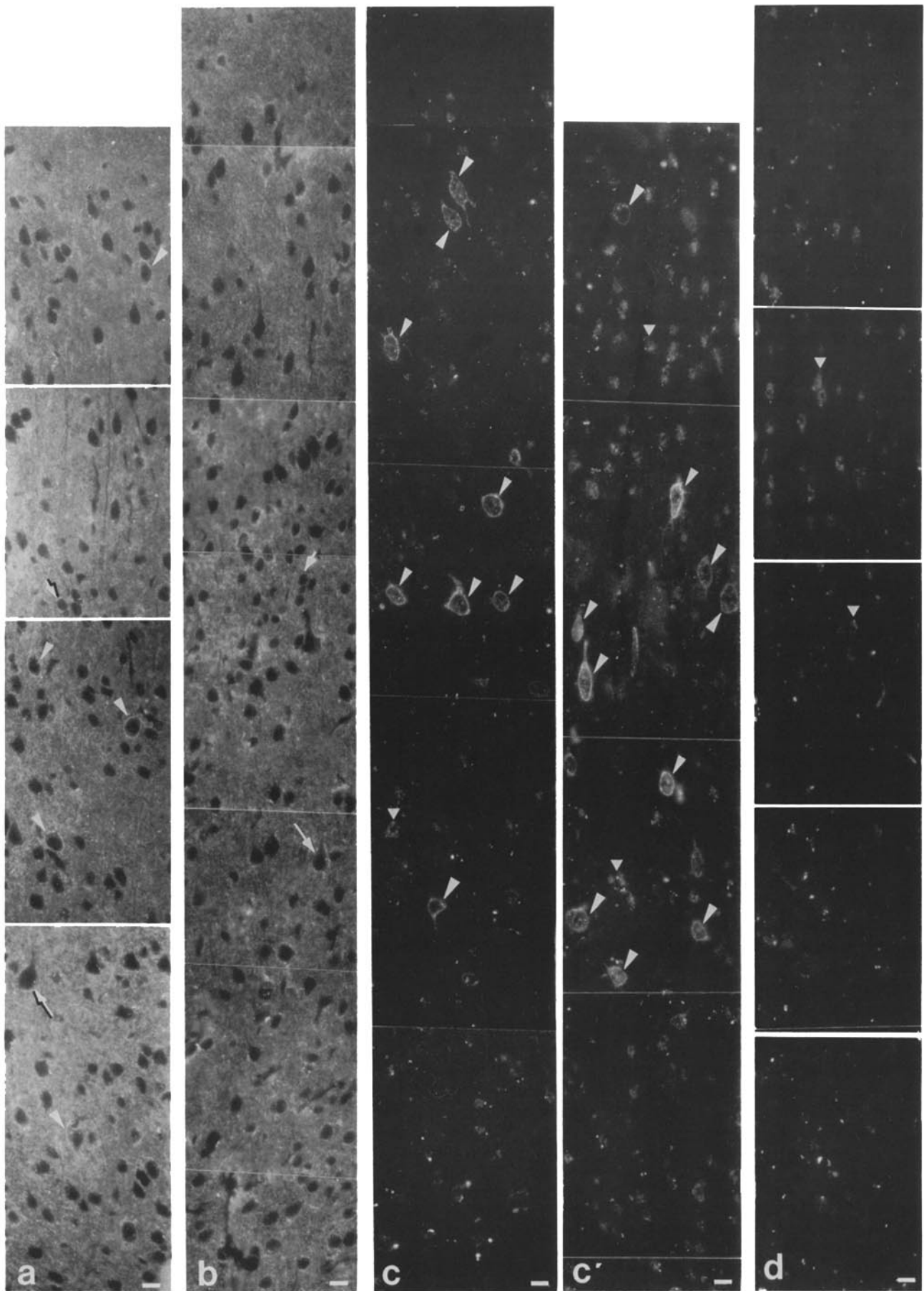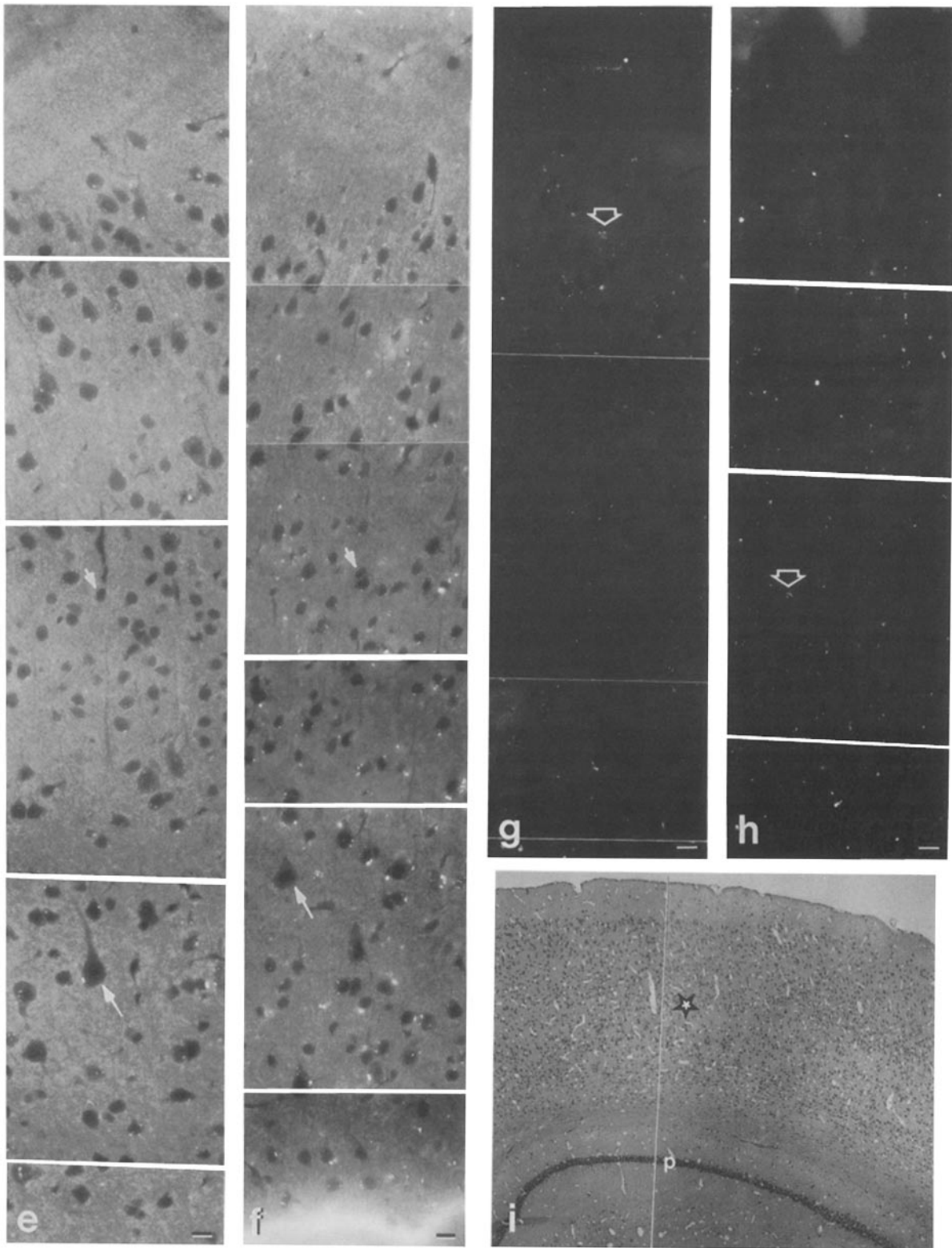
Figure 6. Immunofluorescence localization of ankyrin isoforms on cryosections of normal and *nb/nb* neocortex. Coronal cryosections of neocortex from normal and *nb/nb* mice have been labeled with the RBCank antibody (*a* and *b*, respectively), the ankyrin$_{Ro}$-specific antibody (*c–c'* and *d*, respectively), and the ankyrin$_B$ antibody (*e* and *f*, respectively). *c* and *c'* show two different areas of neocortex labeled with the ankyrin$_{Ro}$-specific antibody. *i* is a histologically labeled section indicating the area of neocortex (*asterisk*) opposite to hippocampus pyramidal layer (*p*) from where all immunofluorescence pictures were taken. In control experiments, the first antibody was replaced by rabbit nonimmune Ig during incubations (*g* and *h* for normal and *nb/nb* tissue, respectively). Long arrowheads point to labeled plasma membranes of neuron cells, short arrows to unlabeled small granule (stellate) neurons, long arrows to unlabeled large pyramidal cells, short arrowheads to nuclei and open arrows to autofluorescing lipofuskin granule within the neuron cell cytoplasm. Bars, 10 μm.

Figure 7. Immunofluorescence localization of ankyrin isoforms on cryosections of normal and *nb/nb* brain stem. Cross cryosections from the middle of brain stem of normal and *nb/nb* mice have been labeled with the RBCank antibody (*a* and *b*, respectively), the ankyrin$_{Ro}$-specific antibody (*c* and *d*, respectively), and the ankyrin$_B$ antibody (*e* and *f*, respectively). Insets in *a* and *c* show neuron cells from a different region of the same section; pictures shown in both insets are from homologous areas. In control experiments, the first antibody
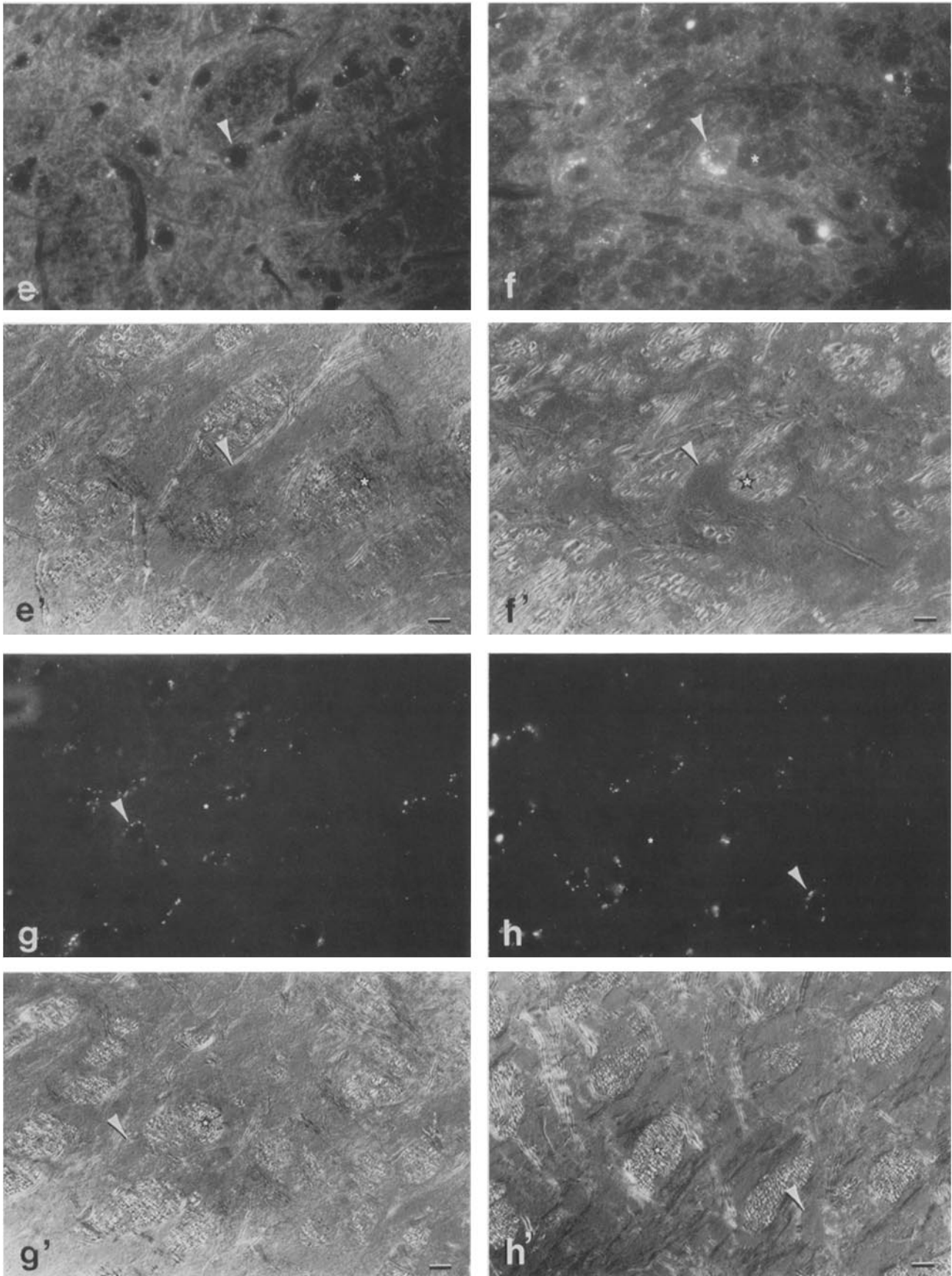
was replaced by rabbit nonimmune Ig during incubations (*g* and *h* for normal and *nb/nb* tissue, respectively). Arrowheads point to plasma membranes of neuron cells and asterisks show cross-sectioned bundles of myelinated axons. *a'–h'* are DIC micrographs corresponding to immunofluorescence micrographs *a–h*. Bars, 10 μm.

pre-mRNA not altered by the *nb* mutation, cannot be excluded. Another interesting observation was that, at least in spinal cord of *nb/nb* mice, labeling of initial axonal segments with the RBCank antibody was observed, in contrast with loss of labeling of the neuron cell body. This observation suggests that the same ankyrin isoform is present at two membrane domains showing homologous organization, namely the nodes of Ranvier and the initial axonal segments of neuron cells (reviewed in Waxman and Ritchie, 1985).

An interesting question for future study is the state of spectrins in the nervous system, and in particular in the mutant mice. In this study, the relative amounts of the brain spectrin (fodrin) polypeptides between normal and mutant mice seemed to remain unchanged in all parts of the nervous tissue examined, as judged by the Coomassie blue–stained polyacrylamide gel (Fig. 1 *A*). Ankyrin$_B$ remains also unchanged in the mutant nervous system. Previous studies in our laboratory showed that ankyrin$_B$ and erythrocyte ankyrin bind with higher affinity to fodrin and erythrocyte spectrin, respectively (Davis and Bennett, 1984*b*). These observations would allow us to speculate that each form of ankyrin preferentially interacts and therefore coexists with the corresponding form of spectrin within cells. Bodine et al. (1984) showed that erythrocyte spectrin is reduced by 50% in the mutant *nb/nb* erythrocytes, as a result of partially impaired assembly on the membrane due to ankyrin deficiency. It has been shown as well that erythroid β–spectrin gene is expressed in the nervous system (Winkelmann et al., 1990). Previous localization studies in avian and murine nervous tissue showed that erythroid β-spectrins were associated with neuronal cell bodies and dendrites (Lazarides et al., 1984; Riederer et al., 1986). It is of interest that such a distribution would correlate with our results on ankyrin$_{Ro}$ distribution. Taken together these observations lead to the speculation that, in the nervous system, fodrin might selectively associate to ankyrin$_B$ and erythroid spectrin to ankyrin$_{Ro}$. As a consequence, the mutant nervous tissue might be partially deficient in erythroid spectrin as well.

The data of this study strongly suggest that at least two different forms of ankyrin$_R$ are expressed within neurons. An interesting question arises as to how these proteins are targeted to completely different subcellular compartments, such as the cell body and the nodes of Ranvier. Identification of the isoform at the nodes of Ranvier is expected to provide insight into mechanisms involved in sorting and targeting of unique components of specialized membrane domains, and consequently in the establishment of these domains.

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