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Expression and Assembly of the Erythroid Membrane-Skeletal Proteins Ankyrin (Goblin) and Spectrin in the Morphogenesis of Chicken Neurons

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The membrane-skeleton of adult chicken neurons in the cerebellum and optic system is composed of polypeptides structurally and functionally related to the erythroid proteins spectrin and ankyrin, respectively. Neuronal spectrin comprises two distinct complexes that share a common α subunit (M_r 240,000) but which have structurally distinct polymorphic subunits ($\beta'\beta$ spectrin; M_r 220/225,000; γ spectrin, M_r 235,000); the brain-specific form ($\alpha\gamma$ spectrin or fodrin) and an erythrocyte-specific form ($\alpha\beta'\beta$ spectrin). Two structurally related isoforms of ankyrin have also been identified and are termed α (M_r 260,000) and β (M_r 237,000) ankyrin. Immunofluorescence demonstrates that the variants of spectrin and ankyrin, respectively, have different distributions within neurons. On the one hand, $\alpha\gamma$ spectrin and β ankyrin are present throughout the neuron, in the perikaryon, dendrites, and axon, whereas $\alpha\beta'$ spectrin and α ankyrin are localized exclusively in the perikaryon and dendrites where they are actively segregated from $\alpha\gamma$ spectrin and other components of axonal transport. This asymmetric distribution of spectrin and ankyrin isoforms is established in distinct stages during neuronal morphogenesis. Early in cerebellar and retinal development, $\alpha\gamma$ spectrin is expressed in mitotic cells. Subsequently β ankyrin and $\alpha\gamma$ spectrin are co-expressed in postmitotic cells and gradually accumulate on the plasma membrane in a uniform pattern throughout the neuron during the phase of cell growth. At the onset of synaptogenesis and the cessation of cell growth, their levels of synthesis decline sharply while the assembled proteins remained as stable membrane components. Concomitantly, there is a dramatic induction in the accumulation of α ankyrin and $\alpha\beta'$ spectrin, whose assembly is limited to the plasma membrane of the perikarya and dendrites. These results demonstrate that two successive, developmentally regulated programs of ankyrin and spectrin expression and patterning on the plasma membrane are involved in the assembly of the spectrin-based asymmetry in the neuronal membrane-skeleton, and that their asymmetric distribution is actively maintained throughout the life of the neuron.

Key words: ankyrin, spectrin, membrane assembly, neuronal differentiation

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Morphological and electrophysiological studies on neurons in vivo and in vitro have demonstrated a striking structural and physiological asymmetry in these cells. At one end of the neuron is the perikaryon and associated dendrites, which receive electrical and chemical signals that impinge onto the postsynaptic terminals located on their plasma membranes; the perikaryon also contains the nucleus and all the protein-synthesizing machinery of the cell. These signals are relayed down the axon, which extends away from the perikaryon, by the unidirectional propagation of action potentials and the axonal transport of proteins and organelles from the perikaryon to the distal end of the axon, where the presynaptic terminal is located. Hence, the asymmetry of the neuron is defined by the morphological and physiological differences that exist between the perikaryon, dendrites, and axon, respectively. This asymmetry is established during neuronal morphogenesis in at least two discrete stages. The first stage involves the outgrowth of the axon and dendrites, which occurs during the phase of cell growth following withdrawal of the cell from the proliferative cell cycle. Subsequently, upon contact of the growth cone of one neuron with the perikaryon or dendrites of another (synaptogenesis), cell growth ceases and the phase of terminal differentiation is initiated. The concomitant gradual formation of the pre- and postsynaptic terminals upon synaptogenesis marks the second stage in the establishment of the structural and physiological asymmetry of the neuron.

Although the molecular mechanisms which regulate these morphogenetic events are largely unknown, the striking structural asymmetry of the mature neuron suggests that the assembly of the membrane-skeleton may play an important role in neuronal morphogenesis. Furthermore, since neurons, like many other cell types, contain a subcortical cytoskeletal network of actin filaments, a mechanism for restricting the distribution of proteins to different domains on the plasma membrane in the mature neuron may also involve elements of the cytoskeleton and, in particular, the membrane-associated forms of actin and actin-binding proteins that mediate the attachment of actin to the plasma membrane.

To gain an insight into the molecular basis of neuronal morphogenesis and the role of the membrane-skeleton in the maintenance of the structural and physiological asymmetry of the neuron, we have recently analyzed biochemically and immunologically the molecular composition of the membrane-skeleton of neurons in the developing and adult chicken cerebellum and optic system. As a basis for these studies, we have sought to identify specific polypeptides that are structurally and functionally related to the well-defined components of the erythrocyte membrane-skeleton, in which the molecular interactions between integral membrane proteins and structural proteins in the subcortical cytoplasm have been characterized extensively.

In the erythrocyte, the membrane-skeleton consists predominantly of actin and the actin-binding protein spectrin, the latter accounting for 75% of the protein mass [50]. Spectrin is composed of two nonidentical polypeptides, termed α spectrin (M_r 240,000) and β spectrin (M_r 220,000) that self-associate to form an $(\alpha\beta)_2$ tetramer [for references, see 8,16]. This spectrin tetramer binds to and crosslinks filamentous (F) actin [17] in the subcortical cytoplasm and, through the β subunit, is attached to another polypeptide, termed ankyrin, which is itself directly bound to the transmembrane-orientated anion transporter [9–11,75]. In avian erythrocytes, the structural and functional analogue of ankyrin is a protein termed goblin [53,56], which has been characterized previously as a phosphoprotein whose phosphorylation is hormone-sensitive and is correlated quantitatively with an increase in Na^+ -K^+ -cotransport

[2,3,7]. Additionally, spectrin tetramers may interact with the plasma membrane through a polypeptide termed protein 4.1 [for references see 8,16,46]. Protein 4.1 interacts simultaneously with spectrin and actin and greatly increases the affinity of the latter two polypeptides for each other [for references see 8,16] and further mediates their attachment to the plasma membrane by interacting with the membrane-bound protein glycophorin [4]. These specific protein interactions form a highly crosslinked protein matrix which provides tensile strength to the plasma membrane and restricts the lateral mobility of certain membrane proteins in the plane of the membrane.

The polypeptides identified in our studies on the neuronal membrane-skeleton are the analogues in chicken neurons of chicken erythrocyte spectrin and ankyrin (goblin). Using a combination of biochemical and immunological techniques, we have demonstrated the expression of discrete isoforms of spectrin and ankyrin in cerebellar and retinal neurons, and the existence of a spectrin-based asymmetry in the molecular composition of the membrane-skeleton of these two neuronal systems. Furthermore, by analyzing the synthesis and accumulation of ankyrin and spectrin during development we have demonstrated that the assembly of different isoforms occurs in distinct steps during neuronal morphogenesis. Together, the results obtained provide for the first time experimental evidence to support the hypothesis that the membrane-skeleton plays an important role in neuronal morphogenesis and in the maintenance of the morphological and physiological asymmetry of the mature neuron.

IDENTIFICATION OF COMPONENTS OF THE MEMBRANE-SKELETON IN ADULT CHICKEN NEURONS

Two Forms of Spectrin are Expressed in Neurons of Adult Chicken Cerebellum and Optic System

Immunoautoradiography of SDS-solubilized extracts of adult chicken cerebellum with a polyclonal antiserum specific for the α subunit of chicken erythrocyte spectrin has revealed the presence of a polypeptide with a apparent molecular weight (M_r 240,000) and isoelectric point similar to that of erythrocyte α spectrin [12,19,29,62,64]. Two-dimensional peptide mapping of chymotryptic iodopeptides derived from chicken erythrocyte α spectrin and from this serologically related polypeptide in chicken cerebellum has demonstrated that these polypeptides are structurally indistinguishable, indicating that they share homologous, if not identical, primary structures [31,58]. However, mammalian erythrocyte α spectrin has a different two-dimensional peptide map to the serologically related polypeptide from mammalian brain [12], indicating that mammalian erythrocyte α spectrin has diverged structurally during evolution from its counterpart in brain and avian erythrocytes.

Under certain experimental conditions, the chicken α spectrin antiserum coimmunoprecipitates from cerebellar extracts a second polypeptide (M_r 235,000) in equimolar amounts with α spectrin [48,55], which is both serologically and, by peptide mapping, structurally distinct from both the α and the β subunits of erythrocyte spectrin [31,55,58]; this polypeptide is termed γ spectrin [59] (see Fig. 1). Several studies have shown that brain α spectrin and γ spectrin copurify with neuronal plasma membranes as a complex whose conformation is similar to that of erythrocyte $\alpha\beta$ spectrin; this polypeptide has been referred to in the past as fodrin or brain spectrin [12,48]. The brain aγ-spectrin complex is a rod-shaped molecule comprising

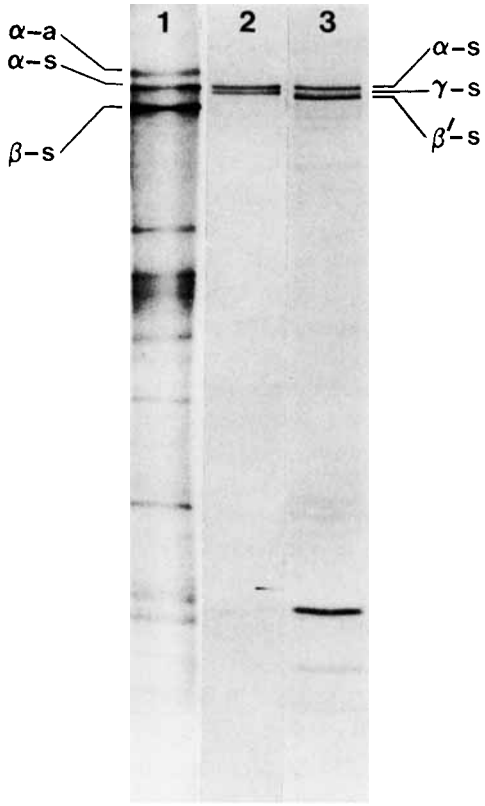

Fig. 1. Identification of two distinct spectrin complexes in neurons of adult chicken retina. A 2-day-old chicken was anesthetized with halothane (1-2%) mixed with nitrous oxide/oxygen (2:1). Approximately 1 mCi [^{35}S]methionine in 10 μl phosphate-buffered saline was pressure-injected into one eyeball using a glass micropipette. The chicken was sacrificed 96 hr after injection. The retina was removed and homogenized in an all-glass Dounce homogenizer in 2 ml of 0.5% Empigen, 10 mM Tris-HCl, pH 7.5, 2 mM EDTA, 1 mM EGTA, 1 mM DTT, 130 mM NaCl, and then stirred at 4°C for 2 hr. Insoluble material was removed by centrifugation at 50,000g for 10 min. The supernatant was divided into two fractions; 2 μl chicken erythrocyte α-spectrin antiserum [64] was added to one fraction and 2 μl chicken erythrocyte β-spectrin antiserum [55] was added to the second. The immunoprecipitation reaction was allowed to proceed overnight at 4°C. The immune complexes from each fraction were isolated with protein A-bearing Staphylococcus aureus, and analyzed by SDS-12.5% polyacrylamide gel electrophoresis and fluorography. The resulting fluorogram is shown here. Lane 1) ^{35}S-methionine-labeled total membrane-skeletal proteins from 10-day chick embryo erythroid cells. Lane 2) α-spectrin immunoprecipitate of ^{35}S-methionine-labeled retina. Lane 3) β-spectrin immunoprecipitate of ^{35}S-methionine-labeled retina. α-s, α spectrin; γ-s, γ spectrin; β′-s, β′ spectrin; β-s, β spectrin; α-a, α ankyrin.

a tetramer formed by the head-on association of two identical dimers, composed of a 1:1 molar ratio of α:γ spectrin [12,30]. Furthermore, the brain spectrin tetramer also binds to and crosslinks F actin [12,30].

Recently, we have demonstrated that neurons of the adult chicken cerebellum and optic system also contain an erythroid form of spectrin [43,44,45]. This second form of spectrin in neurons was identified using a polyclonal antiserum specific for the β subunit of chicken erythrocyte spectrin [55]. Previous studies had shown that this antiserum reacts specifically with chicken erythrocyte β spectrin (M_r 220,000) and a minor component (M_r 230,000) of chicken erythrocyte plasma membranes with which β spectrin shares antigenic determinants but which is antigenically and structurally distinct from α spectrin, γ spectrin, and ankyrin (goblin); this polypeptide is termed β' spectrin [55,58]. Immunoautoradiography of SDS-solubilized extracts of adult chicken cerebellum [43] and retina [45] with the β-spectrin-specific antiserum has revealed the presence of two crossreacting polypeptides. The predominant component has an electrophoretic mobility similar to that of erythrocyte β' spectrin, while the minor component has a slightly faster relative mobility which is similar to that of β spectrin. Two-dimensional mapping of chymotryptic iodopeptides has demonstrated a striking homology between neuronal and erythroid β' spectrin, indicating that they have similar, if not identical, primary structures [45]. The relatively low amounts of the minor component of neurons that crossreacts with the β-spectrin antiserum has, thus far, precluded a similar structural comparison of this polypeptide with its erythroid counterpart.

Immunoprecipitation with β-spectrin antiserum of adult chicken cerebellar and retinal proteins extracted in the presence of the Zwitterionic detergent Empigen demonstrates that β' spectrin is present as a complex with equimolar amounts of α-spectrin, which is coimmunoprecipitated with β' spectrin under these conditions (Fig. 1). Little or no γ spectrin is coimmunoprecipitated with the $\alpha\beta'$-spectrin complex (Fig. 1).

Taken together, these results demonstrate clearly that two forms of spectrin are expressed in adult chicken neurons, a brain-specific form, $\alpha\gamma$ spectrin, and an erythroid form, $\alpha\beta'$ spectrin (Fig. 1). Furthermore, $\alpha\gamma$ and $\alpha\beta'$ spectrin appear to be distinct complexes which share a common α subunit.

Expression of Two Ankyrin (Goblin) Isoforms in the Neurons of Adult Chicken Cerebellum and Retina

In view of the fact that an erythroid form of spectrin is present in chicken neurons, we have sought to determine whether polypeptides serologically and structurally related to erythrocyte ankyrin are also present in neurons [57]. Indeed, a polyclonal antiserum raised against erythrocyte ankyrin (goblin) [56] was shown to react with two polypeptides in SDS-solubilized extracts of adult chicken cerebellum and retina [57]. The larger polypeptide has an apparent molecular weight of 260,000 (termed α ankyrin), which is similar to that of the predominant form of ankyrin (goblin) in chicken erythrocytes. The smaller polypeptide has an apparent molecular weight of 237,000 (termed β ankyrin) similar to that of a minor component of chicken erythrocyte membranes with which α ankyrin shares antigenic determinants, but which is antigenically distinct from α, γ and β spectrin [56,57]. Both α and β ankyrin in neurons are characterized by hormone-dependent phosphorylation and copurify

with a plasma membrane fraction from brain [57], similar to their counterparts in erythrocytes [2,7,56,57]. In addition, two-dimensional peptide mapping has revealed extensive homologies between the erythroid and neuronal polypeptides, indicating that they have similar primary structures [57]. Thus, these results demonstrate that the ankyrin polypeptides in chicken cerebellum and retina are both structurally and biochemically related to erythrocyte ankyrin.

ASYMMETRY IN THE MOLECULAR COMPOSITION OF THE NEURONAL MEMBRANE-SKELETON

$\alpha\gamma$ and $\alpha\beta'$ Spectrin Have Different Distributions in the Same Neuron

The distribution of $\alpha\gamma$ and $\alpha\beta'$ spectrin in the neurons of adult chicken cerebellum and optic system has been determined by indirect immunofluorescence microscopy [43,45]. Immunofluorescence on frozen sections of cerebellum with the α-spectrin-specific antiserum has revealed that this antigen is present in all three layers of the cerebellar cortex and in all discernible perikarya, dendrites, and axonal processes that populate the molecular, Purkinje cell, and granular cell layers [43]. Since γ spectrin is present in an equimolar complex with α spectrin (see above), we assume that γ spectrin has an identical distribution to that of α spectrin (see also 48).

In contrast to this uniform distribution of $\alpha\gamma$ spectrin throughout the cerebellar cortex, immunofluorescence with the β-spectrin-specific antiserum has revealed that this antigen has a more restricted distribution. Comparison of alternate sections stained with β-spectrin antiserum and with Nissl stain has revealed that the perikarya of granular cells and Purkinje cells show bright fluorescence in association with the plasmalemma, which, in the case of the Purkinje cells, is also detected in the initial portion of their dendritic trunks that extend into the molecular layer [43]. The rest of the molecular layer does not show any substantial fluorescence above that of the preimmune β-spectrin serum, indicating that $\alpha\beta'$ spectrin is not present in the numerous axonal and dendritic processes that populate this layer of the cerebellum. In addition, processes in the white matter were shown to be uniformly negative with the β-spectrin antiserum, indicating that the axonal processes of mossy and climbing fibers and those of Purkinje cells do not contain $\alpha\beta'$ spectrin but do contain $\alpha(\gamma)$ spectrin (see above). A notable exception in the white matter were the perikarya of neurons of the cerebellar nuclei, which stained strongly with the β-spectrin antiserum. As in the case of the Purkinje cells, β-spectrin-specific fluorescence was associated with the periphery of these cells and was also detected a short distance into the main processes emanating from the perikarya [43].

These results demonstrate that $\alpha\gamma$ spectrin is present throughout the neuron in all discernible types of neurons, whereas the distribution of $\alpha\beta'$ spectrin is restricted to the perikarya of certain cerebellar neurons. That the differential distribution of these two forms of spectrin is a general feature of neurons and is not restricted to those present in the cerebellum was demonstrated by our analysis of the distribution of $\alpha\gamma$ and $\alpha\beta'$ spectrin in the neurons of the adult chicken optic system [45]. As in the cerebellum, α spectrin was detected by immunofluorescence in all discernible layers of the retina including the axons of the optic nerve fibers; the perikarya of ganglion cells; numerous processes in the inner plexiform layer, which are presumed to be ganglion cell dendrites and amacrine cell processes; the perikarya of amacrine,

bipolar, and horizontal cells in the inner nuclear layer; cell processes in the outer plexiform layer; and the perikarya of rods and cones in the outer nuclear layer. A notable exception was the absence of any reactive forms in the bacillary layer, which contains the outer segments of the rods and cones [45].

Immunofluorescence with the β-spectrin-specific antiserum on frozen sections of retina revealed that, as in the cerebellum, $(\alpha)\beta'$ spectrin has a restricted distribution. β'-spectrin fluorescence was detected at the plasma membrane of only the perikarya of ganglion cells, the axonal processes of optic nerve fibers within the retina (but not in the optic nerve), and a subset of the processes in the inner plexiform layer close to the ganglion cell layer, which are presumed to be the dendritic processes of ganglion cells that extend into the inner plexiform layer in the avian retina [5,60]. The perikarya and processes of amacrine, bipolar, and horizontal cells were uniformly negative. A notable exception, however, were the perikarya of rods and cones in the outer plexiform layer which were positive with β-spectrin antiserum [45].

Distinct Membrane-Skeleton Domains in Neurons

The finding that there is a differential distribution of $\alpha\gamma$ and $\alpha\beta'$ spectrin in adult chicken cerebellar and retinal neurons demonstrates for the first time a distinctive asymmetry in the molecular composition of the membrane-skeleton of neurons, which appears to exist between the perikaryon and dendrites on the one hand and the axon on the other. More recently, an asymmetric distribution in neurons of several other structural proteins has been noted. These include the microtubule-associated proteins -1 and -2 (MAP-1 and MAP-2), of which MAP-1 is present throughout the neuron, whereas MAP-2 is localized to the perikaryon and dendrites [26,38]; the neurofilament subunits, of which the M_r 68,000 and M_r 170,000 subunits are present throughout the neuron, whereas the M_r 195,000 subunit is localized in the axon [37; see also 78]; and isoforms of tubulin, which show a graded distribution between the perikaryon and the distal end of the axon [33]. These results show that the asymmetric distribution of $\alpha\gamma$ and $\alpha\beta'$ spectrin in neurons is not unique but is a feature of several other structural proteins as well, which together suggest that distinct domains exist in the neuronal membrane-skeleton.

ESTABLISHMENT OF ASYMMETRY IN THE MEMBRANE-SKELETON DURING NEURONAL MORPHOGENESIS

The fact that there is an asymmetry in the molecular composition of the neuronal membrane-skeleton raises the question as to the sequence of developmental events involved in its assembly during neuronal morphogenesis, particularly with respect to the phases of cell growth and terminal differentiation. In the chick embryo, neuronal morphogenesis occurs in two major phases. First, upon withdrawal of the cells from the proliferative cell cycle, there is a dramatic outgrowth of their axons and dendrites; this constitutes the phase of cell growth. Subsequently, on about embryonic day 15 in both retinal and cerebellar neurons, the cells commence synaptogenesis and begin the phase of terminal differentiation; at the same time there is a gradual cessation of cell growth [for references on cerebellum, see 40,54,71; and retina, see 5,18, 39,44,60,61, 63,70,73] (see Fig. 2A).

By analyzing the temporal and spatial expression during neuronal morphogenesis of each spectrin and ankyrin isoform, we sought to determine the steps in the assembly of these proteins during the phases of cell growth and terminal differentiation.

Developmentally Regulated Expression and Patterning on the Neuronal Plasma Membrane of Spectrin and Ankyrin Isoforms During Ontogeny of Chick Cerebellum and Retina

To determine the temporal and spatial accumulation of spectrin and ankyrin isoforms during neuronal morphogenesis, a combination of immunofluorescence and biochemical techniques has been used in parallel on the developing chick cerebellum and retina [44,45,57].

For immunofluorescence, frozen sections of the cerebellar cortex from chicken embryos at embryonic days 17–21 were used. The chick cerebellum at these stages of embryogenesis is ideal for the analysis of neurons at all phases of differentiation. In the same frozen section, cells in the following phases can be detected, from the outside to the inside of the cortex: mitotic cells in the external granular cell layer; postmitotic cells (mostly granule cells) migrating through the molecular layer; and Purkinje and granule cells which have reached their designated position in the cortex (Purkinje cell layer and internal granular cell layer, respectively), and which are undergoing terminal differentiation upon the establishment of synaptic connections with climbing and mossy fibers, respectively [40]. For biochemical analysis of the expression of spectrin and ankyrin isoforms during neuronal morphogenesis, both the accumulation at steady state and the level of synthesis were determined. The trend in accumulation was analyzed by immunoautoradiography of SDS-solubilized extracts of cerebellum and retina and the levels of synthesis by ^{35}S-methionine incorporation in vitro into whole retina followed by immunoprecipitation and fluorography (see Fig. 2, and [57]).

Fig. 2. Developmentally regulated changes in the levels of synthesis and rates of accumulation of ankyrin and spectrin isoforms during neuronal morphogenesis. A) Changes in the average widths of layers in the cerebellar cortex of the chick during embryogenesis; the width of the external granular (EGL) layer is an indication of the proportion of mitotic cells, whereas that of the internal granular (IGL) layer indicates the proportion of terminally differentiated cells; ML, molecular layer: plotted from data in Saetersdal [66] and Addison [1]; see also Jacobson [40]. B) Developmentally regulated decline in the level of $\alpha\gamma$-spectrin synthesis during retinal development. Whole retinas from embryonic day 12–21 chicks were labeled radioactively with ^{35}S-methionine in vitro. $\alpha\gamma$ Spectrin was co-immunoprecipitated with α-spectrin-specific antiserum and analyzed by SDS-12.5% polyacrylamide gel electrophoresis and fluorography. Densitometric analysis of the fluorogram was performed and the resulting values expressed as a percentage of that on embryonic day 12. C and D) Developmentally regulated accumulation of ankyrin and spectrin isoforms during chick cerebellar ontogeny. SDS-solubilized extracts of embryonic cerebellum (days 13–21) were separated on SDS-12.5% polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with ankyrin, α- and β-spectrin antisera, respectively. The immunocomplexes were localized with ^{125}I-labeled protein A and subsequent autoradiography. Densitometric analysis of the autoradiograms was performed and the values normalized to the DNA concentration of the initial extract and then exposed as a percentage of the amount present on embryonic day 13. $\alpha\gamma$-s, $\alpha\gamma$ spectrin (○-○); β-s, β spectrin (●-●); α-a, α ankyrin (▲-▲); β-a, β ankyrin (△-△). Arrow indicates day of hatching (H). Data from [57].

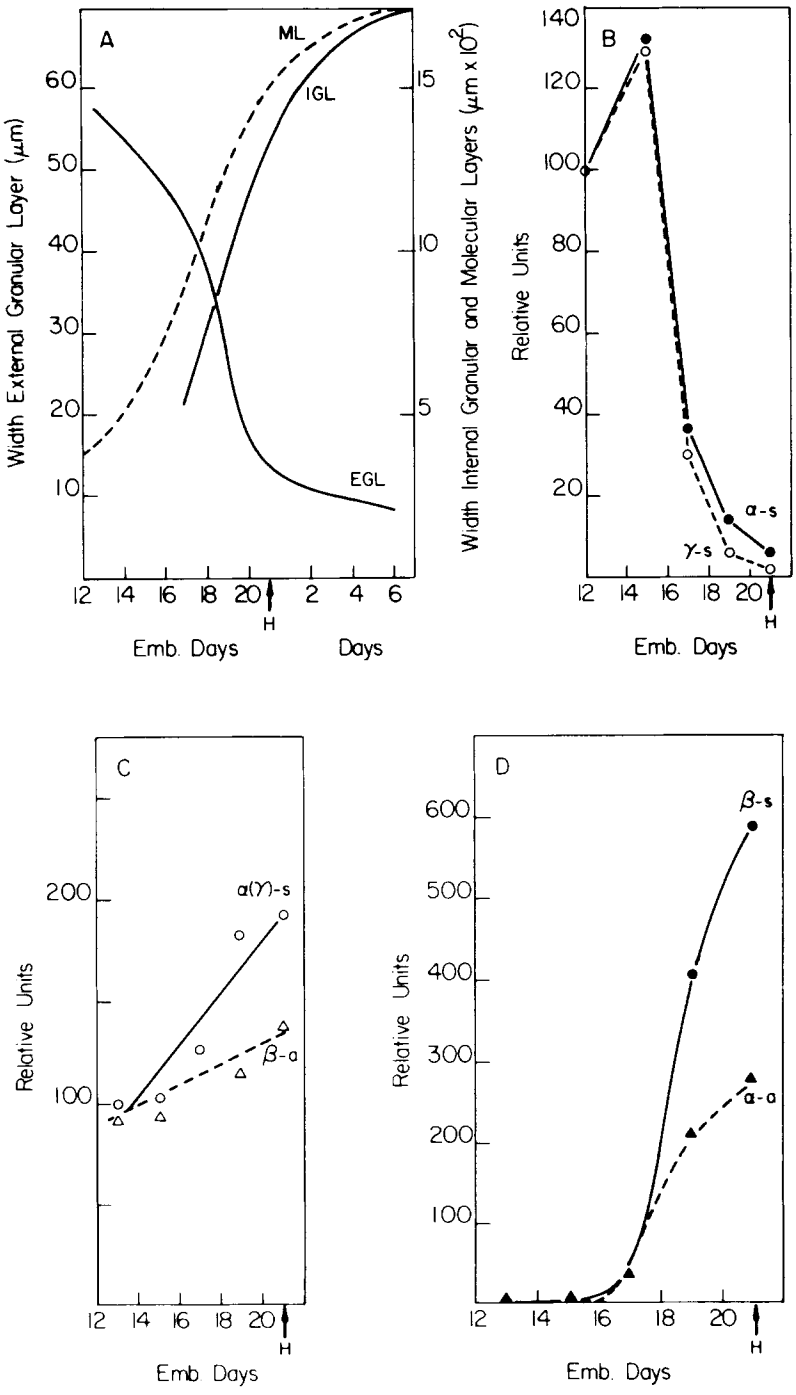
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Fig. 2.

Mitotic cells of embryonic chicken cerebellum and retina express and accumulate at steady state $\alpha\gamma$ spectrin, but not $\alpha\beta'$ spectrin and little or no ankyrin, as shown by indirect immunofluorescence [44,45,57]. However, in the same frozen sections, postmitotic cells exhibit fluorescence with the ankyrin antiserum. This suggests that there is an induction in the expression and/or accumulation of ankyrin upon withdrawal of the cells from the proliferative cell cycle. Immunoautoradiography of a day-13 chick embryo cerebellum, which contains mostly mitotic and postmitotic cells [40], has revealed that the ankyrin isoform expressed at this stage is β ankyrin and that little or no α ankyrin is present [57]. A similar result was obtained from the retina at the same embryonic stage (our unpublished results).

Analysis of the levels of synthesis of $\alpha\gamma$ spectrin in the retina has shown an increase between embryonic days 12 and 15. Subsequently, a sharp decline in the levels was detected such that by the time of hatching the level of $\alpha\gamma$ spectrin synthesis was reduced to approximately 5% of the level observed on embryonic day 12 (Fig. 2B) [see 57]. In contrast, immunoautoradiography of embryonic cerebella and retinas demonstrates a gradual increase in the steady state amount of $\alpha\gamma$ spectrin (Fig. 2C) [see 57], indicating that as the level of synthesis declines there is a concurrent stabilization of newly synthesized protein against proteolytic degradation. During the same period of cerebellar and retinal development, a gradual accumulation of β ankyrin is also detected (Fig. 2C). Although ^{35}S-methionine incorporation into β ankyrin was detected during retinal ontogeny, the amounts were too low to quantify. However, we were able to observe a qualitative decline in the incorporation of ^{35}S-methionine into β ankyrin between embryonic day 13 and day 21; in the adult, little or no β-ankyrin synthesis was detected in vivo [57]. Thus, these results suggest that β ankyrin also exhibits a pattern of decreased synthesis and concomitant stabilization in parallel with that of $\alpha\gamma$ spectrin during the later stages of neuronal differentiation.

The sharp decline in the level of $\alpha\gamma$-spectrin and β-ankyrin synthesis on embryonic day 15 coincides with a dramatic induction in the accumulation in the embryonic cerebellum of two new isoforms of ankyrin and spectrin, namely α ankyrin and β' spectrin. Immunofluorescence of developing cerebellum with β-spectrin antiserum has revealed that, throughout all stages of cerebellar ontogeny, mitotic cells and presumptive postmitotic granular cells that are migrating through the molecular layer and Purkinje cell layer exhibit no fluorescence [44]. Furthermore, there appears to be no β-spectrin fluorescence in the few presumptive granule cells that have migrated past the Purkinje cell layer prior to embryonic day 15/17 and which are forming the internal granular cell layer. The first indication of β-spectrin accumulation is on embryonic day 15/17, when small foci of β-spectrin fluorescence were detected at the plasma membrane of the perikarya of granule cells below the Purkinje cell layer. The degree of this fluorescence became more prominent by embryonic day 19, and by hatching (day 21) was found to be essentially indistinguishable from that in the adult cerebellum [43,44]. Before the onset of granule cell migration on embryonic day 15, the Purkinje cells have already migrated and lined up to form a distinct layer [40]. However, β-spectrin fluorescence was not detected in the perikarya of these cells until embryonic day 16/17, and approximately 24 hr before it was detected in the granule cells [44]. In addition, β-spectrin fluorescence was detected in the perikarya of the neurons of the cerebellar nuclei, but also only subsequent to embryonic day 16/17.

In the developing retina, we also detected the expression of $\alpha\gamma$ spectrin throughout neuronal differentiation in all developing layers and the developmentally regulated appearance of β' spectrin on embryonic day 17 at the plasma membrane of the perikarya and dendrites of ganglion cells and the perikarya of rods and cones [45].

Thus, in contrast to the constitutive expression of $\alpha\gamma$ spectrin and β ankyrin, the expression of β' spectrin and α goblin is developmentally regulated. This was demonstrated more directly by immunoautoradiography of SDS-solubilized extracts of day-13–21 embryonic chick cerebella [57] (see also Fig. 2D). On embryonic days 13–15, little or no β' spectrin or α ankyrin is detected. However, beginning on embryonic day 15/17, there is a dramatic induction in the accumulation of β' spectrin and α ankyrin, whose relative level of accumulation appears to reach a plateau by embryonic day 21 (hatching) [57]. As in the case of β ankyrin (see above), we were only able to determine qualitatively the levels of β' spectrin and α ankyrin synthesis in the developing retina. The results indicated that there was little or no synthesis of β' spectrin and α ankyrin in neurons on embryonic day 13 but detectable synthesis of both polypeptides by embryonic day 21, and also in the adult [57]. This suggests that, contrary to the decreasing levels of $\alpha\gamma$ spectrin and β ankyrin synthesis during the later stages of neuronal morphogenesis, there is an induction in those of β' spectrin and α ankyrin.

Two Successive Developmentally Regulated Programs of Protein Expression and Patterning on the Plasma Membrane are Involved in the Assembly of the Neuronal Membrane Skeleton

Our analyses of spectrin and ankyrin isoform expression and distribution in neurons during cerebellar and retinal ontogeny provide, for the first time, an insight into the sequence of molecular events involved in the temporal and spatial assembly of the membrane-skeleton during neuronal morphogenesis. We have hypothesized that there are two successive, developmentally regulated programs of protein expression and patterning on the plasma membrane during neuronal morphogenesis [57] (see Fig. 3). The first program commences upon withdrawal of the cells from the proliferative cell cycle, when $\alpha\gamma$ spectrin and β ankyrin gradually accumulate on the plasma membrane as a uniform pattern throughout the neuron during the phase of cell growth (Fig. 3). Upon synaptogenesis and the cessation of cell growth on embryonic day 15/17 [40], the levels of synthesis of $\alpha\gamma$ spectrin and β ankyrin decline rapidly but their steady state amounts continue to increase, indicating a down-regulation in the first program with a concurrent stabilization of newly synthesized proteins assembled onto the plasma membrane (Fig. 3). Concomitantly, there is an induction in the second program during the phase of terminal differentiation as manifested through the rapid induction in the synthesis and accumulation of $\alpha\beta'$ spectrin and α ankyrin in the perikarya and dendrites of neurons, which, together with $\alpha\gamma$ spectrin, continue to be synthesized in the adult (Fig. 3). This nonuniform pattern of assembly of α ankyrin and $\alpha\beta'$ spectrin is superimposed on the uniform pattern of β ankyrin and $\alpha\gamma$ spectrin assembled during the phase of cell growth, which together establish the asymmetry in the molecular composition of the neuronal membrane-skeleton.

These two patterns of ankyrin and spectrin isoform expression and patterning on the plasma membrane during the phases of cell growth and terminal differentiation appear to be representative of two general programs of structural protein expression

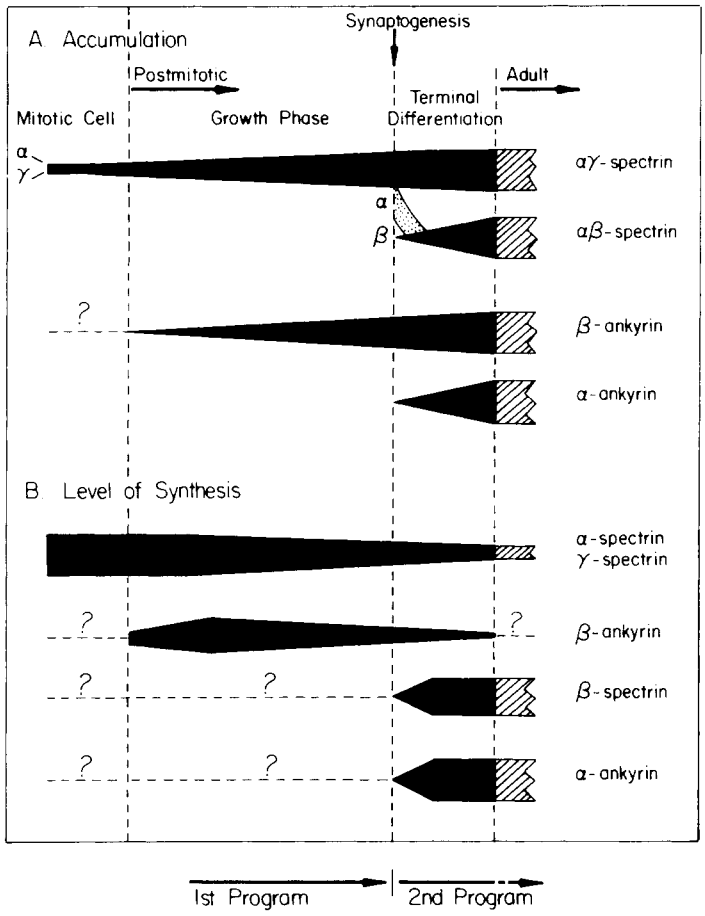

Fig. 3. Schematic representation of the developmentally regulated changes in the levels of synthesis and rates of accumulation of isoforms of ankyrin and spectrin during neuronal morphogenesis. The wedges are not drawn to scale and thus do not represent the relative amounts of each isoform at a given stage. The hatched areas indicate continued synthesis or presence at steady state of an ankyrin or spectrin isoform in the adult.

during neuronal morphogenesis. First, during the phase of cell growth, the gradual accumulation of β ankyrin and $\alpha\gamma$ spectrin is paralleled by an increase in the number of neurofilaments in direct proportion to the increase in cross-sectional area of the axon [13,27,39,77,78, see also 69]. At the same time, there is a decrease in the size of the soluble pools of tubulin and actin [68], presumably reflecting an increase in the number of microtubules and microfilaments in growing axons. Second, upon the onset of synaptogenesis and the cessation of cell growth, there is a sharp decline in the levels of tubulin and actin mRNA [15,68] and their levels of synthesis [68] as shown here also for β ankyrin and $\alpha\gamma$ spectrin, indicating a general down-regulation in the expression of proteins that may be involved in the outgrowth and stabilization of the axons and dendrites during the phase of cell growth. Concomitantly, during the

phase of terminal differentiation there is an induction in the accumulation of a new set of structural proteins which includes MAP-2 [24,26,38,51] together with α ankyrin and $\alpha\beta'$ spectrin which are localized exclusively in the perikarya and dendrites, where they may modulate the topology of the plasma membrane or other cytoskeletal components in the region of the synapse (see below).

Localization of Specific Structural Proteins to the Perikaryon and Dendrites: Implications for Neuronal Function

The temporal and spatial appearance of α ankyrin and $\alpha\beta'$ spectrin has been shown to coincide remarkably with synaptogenesis in the neurons of the chick cerebellum and retina [44,45,57]. Several other proteins have also been shown to appear in conjunction with neuronal terminal differentiation, including Synapsin I, a synaptic vesicle phosphoprotein [25]; neuronal enolase [67], the glycoprotein Thy 1.1 [6], a 23,000-dalton neuronal mitochondrial-specific protein [36], and MAP-2 [38].

The functional significance of the appearance of α goblin and $\alpha\beta'$ spectrin in the perikarya and dendrites upon synaptogenesis and during subsequent neuronal terminal differentiation is at present unknown. However, since chicken neuronal α spectrin and β' spectrin are structurally homologous to their counterparts in erythrocytes (see above), the expression of $\alpha\beta'$ spectrin may be linked to the requirement of the neuronal plasma membrane to have a function analogous to that in the erythrocyte, namely, to maintain an invariant topological distribution of a discrete population of integral membrane proteins in the perikarya and dendrites, and perhaps in the region of the synapse, by restricting their mobility in the plane of the plasma membrane and thereby segregating them from axonal transport [45].

In addition, it may be significant that α ankyrin in neurons [57] and erythrocytes [7,2,3] and MAP-2 in neurons [72,74,76,79] are the target substrates for phosphorylation by cAMP- and Ca^{++} -dependent protein kinases. In neurons, cAMP and Ca^{++} are important second messengers which mediate, at least in part, the action of neurotransmitter signals that occur primarily on the plasma membrane of the dendrites and perikarya [for a review and references, see 35]. Hence, the specific phosphorylation of α ankyrin and MAP-2 in this way may produce dynamic changes in the membrane-skeleton and thus modulate the morphology of the plasma membrane of the dendrites or perikarya at the level of the synapse. Furthermore, in the avian erythrocyte, the hormone-dependent phosphorylation of goblin (ankyrin) has been shown to be correlated quantitatively with an increase in Na^+ -K^+ cotransport [2]. In view of the role of ionic conductance in the transmission of electrical impulses, the specific phosphorylation of ankyrin may play a regulatory role in this respect also in neurons.

ACTIVE MAINTENANCE OF THE ASYMMETRY IN THE NEURONAL MEMBRANE-SKELETON: IMPLICATION FOR AXONAL TRANSPORT

In the mature neuron, newly synthesized proteins and organelles that are destined for the presynaptic terminal are actively segregated from the perikaryon and transported down the axon. Several recent studies have shown that $\alpha\gamma$ spectrin is a major component of axonal transport [45,47–49], which, given the fact that $\alpha\beta'$ spectrin is localized exclusively in the perikaryon and dendrites, indicates that there is a mechanism for segregating these two forms of spectrin during the process of axonal transport.

Active Segregation of $\alpha\gamma$ Spectrin and $\alpha\beta'$ Spectrin in the Adult Chicken Optic System

The distribution of $\alpha\gamma$ and $\alpha\beta'$ spectrin in the adult chicken optic system has been determined by immunoautoradiography of the retina, optic nerve, and optic tract [45]. The results of this analysis graphically demonstrate the asymmetry in the distribution of these two forms of spectrin in the neuron. As expected from the immunofluorescence results, $\alpha\gamma$ spectrin was detected throughout the optic system, in the retina, optic nerve, and optic tract segments. However, $\alpha\beta'$ spectrin was localized exclusively in the retina, where we had shown previously that it is present predominantly in the perikarya of the ganglion cells (see above) [45].

To determine whether $\alpha\gamma$ and $\alpha\beta'$ spectrin are actively segregated from one another during the process of axonal transport, newly synthesized proteins in the retina were labeled radioactively with ^{35}S-methionine in vivo [45]. Ninety-six hours after labeling was initiated, the chicken was sacrificed and the optic system dissected. The distribution of ^{35}S-methionine-labeled $\alpha\gamma$ and $\alpha\beta'$ spectrin in the retina, optic nerve, and optic tract was then determined by immunoprecipitation and fluorography. The results demonstrated that $\alpha\gamma$ spectrin is present throughout the optic system, confirming that this form of spectrin is a component of axonal transport [45,47]. On the other hand, β' spectrin was detected exclusively in the retina and none was found in the optic nerve or optic tract. Therefore, $\alpha\beta'$ spectrin must be actively segregated from $\alpha\gamma$ spectrin and other components of axonal transport in the adult chicken optic system. Since we detected β' spectrin in only the perikarya of cerebellar neurons, whereas $\alpha\gamma$ spectrin was present throughout the neuron, we assume that $\alpha\beta'$ spectrin is also actively segregated from $\alpha\gamma$ spectrin in these neurons as well.

What is the nature of the mechanism(s) for segregating $\alpha\beta'$ spectrin from $\alpha\gamma$ spectrin and axonal transport, and confining it to the perikaryon? Previously, we hypothesized that one mechanism may be that there exists a high-affinity membrane receptor specific for β' spectrin which drives the stable assembly, and hence accumulation, of $\alpha\beta'$ spectrin in the perikaryon despite the presence of excess γ spectrin. The latter may bind to a different membrane receptor which is not retained in the perikaryon and thus $\alpha\gamma$ spectrin becomes a component of axonal transport. α Spectrin, by virtue of the fact that it can bind to either γ or β' spectrin, will assemble with either subunit in equimolar amounts [45]. This hypothesis was based upon two experimental results. First, comparison of the peptide maps of erythroid ($\alpha\beta$) spectrin and $\alpha\gamma$ spectrin [31,45,58] has shown that these two forms of spectrin differ in the polymorphic subunit (γ and β'/β spectrin) which, at least in erythrocytes, is known to be involved in linking the spectrin-actin complex to the plasma membrane; as noted earlier in the erythrocyte, β spectrin binds to an intermediary protein, ankyrin (goblin [56]; see below), which in turn is linked to a subset of the transmembrane-orientated anion transporters [for reviews see 8,16]. Thus the structural difference in the polymorphic subunits may result in the differential binding of the $\alpha\beta'$ and $\alpha\gamma$ spectrin complexes to specific membrane receptors.

Second, recent studies on the assembly of spectrin in embryonic chick erythroid cells [14,53] and in vitro reconstitution experiments with erythroid spectrin [52] have shown that the molar amount of newly synthesized α spectrin assembled onto the membrane is limited by the amount of β spectrin assembled. Furthermore, the assembly of the $\alpha\beta$-spectrin complex onto the membrane is limited by the availability of newly synthesized ankyrin (goblin) [53]. The binding of $\alpha\beta$ spectrin to ankyrin

and to the membrane results in the stabilization of the assembled complex against proteolytic degradation, whereas unassembled subunits synthesized in excess of their membrane binding sites are degraded in the cytosol [14,53].

In view of the fact that ankyrin isoforms are present in adult chicken neurons, we sought to analyze the possibility that ankyrin plays a role in the active segregation of $\alpha\gamma$ and $\alpha\beta'$ spectrin.

Newly Synthesized α Ankyrin is Not a Component of Axonal Transport: A Possible Role for α Ankyrin in the Active Segregation of $\alpha\gamma$ and $\alpha\beta'$ Spectrin

Immunoautoradiography of the adult chicken optic system with ankyrin antiserum has demonstrated that, similarly to $\alpha\beta'$ and $\alpha\gamma$ spectrin, the α and β ankyrin isoforms have different distributions [57]. α Ankyrin is present exclusively in the retina together with $\alpha\beta'$ spectrin, whereas β ankyrin has a distribution throughout the optic system similar to that of $\alpha\gamma$ spectrin. This suggests that α ankyrin is segregated from axonal transport and is selectively retained together with $\alpha\beta'$ spectrin in the retina. Indeed, analysis of the spatial distribution of ^{35}S-methionine-labeled α ankyrin in the optic system 96 hr after labeling in situ has revealed that it is present almost exclusively in the retina and is absent from the optic nerve and optic tract. Thus, newly synthesized α ankyrin and $\alpha\beta'$ spectrin appear to be cosegregated from newly synthesized $\alpha\gamma$ spectrin and the components of axonal transport. Little or no synthesis of β ankyrin has been detected in the adult chicken retina [57].

Given the fact that α ankyrin and β' spectrin share extensive structural homologies with their counterparts in erythrocytes [45,57], one explanation of the cosegregation of α ankyrin and $\alpha\beta'$ spectrin may be that, as in the erythrocyte, α ankyrin acts as the specific membrane attachment protein for erythroid spectrin. In this respect, it is significant that the molar ratio of newly synthesized α ankyrin:β' spectrin in the retina labeled to steady state has been shown to be approximately 1:2 [57], which is similar to their molar ratio at steady state in chicken [56] and mammalian (for references see [8,16]) erythrocytes. This suggests that there is a relatively tight coupling between the levels of synthesis of α ankyrin and β' spectrin which may result in the rapid and complete saturation of newly synthesized α ankyrin binding sites with $\alpha\beta'$ spectrin and hence the efficient assembly of erythroid spectrin onto the plasma membrane of the perikarya and dendrites. Since we detected the synthesis of only α ankyrin in adult neurons, a consequence of the saturation of all newly synthesized α-ankyrin binding sites with $\alpha\beta'$ spectrin is that newly synthesized $\alpha\gamma$ spectrin would be excluded from the plasma membrane of the perikarya and dendrites and may thus become a component of axonal transport. An implicit assumption here is that α ankyrin has a higher affinity for erythroid spectrin than for brain spectrin. This is supported by the results of several studies which have shown that human erythroid ankyrin binds to erythroid spectrin with a significantly higher affinity than to brain spectrin [12,23].

Although the steady state distributions of $\alpha\gamma$ spectrin and β ankyrin in the adult optic system are also similar, we could detect little or no synthesis of β ankyrin in the adult retina, compared to $\alpha\gamma$ spectrin [57]. This suggests that β ankyrin is relatively resistant to proteolytic turnover and, in the absence of its synthesis in the adult, must have accumulated predominantly during the early stages of neuronal morphogenesis prior to hatching; as noted earlier, studies of the assembly of the erythroid membrane-skeleton have demonstrated that ankyrin assembled onto the plasma membrane is

resistant to proteolytic degradation [53]. Thus, if β ankyrin is a membrane attachment protein for either $\alpha\beta'$ or $\alpha\gamma$ spectrin, it may perform this function only during the phase of cell growth, when it accumulates at steady state uniformly on the plasma membrane of postmitotic neurons. Since $\alpha\gamma$ spectrin is the predominant, if not exclusive, form of spectrin synthesized and assembled onto the plasma membrane during this stage of neuronal morphogenesis, this would result in the saturation of all available β-ankyrin binding sites on the axonal plasma membrane with $\alpha\gamma$ spectrin prior to the onset of accumulation of $\alpha\beta'$ spectrin and α ankyrin; consequently, in the adult axon there would not be any β ankyrin-mediated membrane binding sites for newly synthesized $\alpha\beta'$ or $\alpha\gamma$ spectrin. Additionally, it is possible that in the adult there is a protein(s) which acts as the specific membrane attachment site for $\alpha\gamma$ spectrin during axonal transport in the mature neuron but which is not recognized by the ankyrin antiserum used in these studies. In this respect, it may be significant that the kinetics of $\alpha\gamma$ spectrin movement down the axon show that it is a component of more than one group of transported proteins [47–49], indicating that $\alpha\gamma$ spectrin may have the capacity to bind to several different proteins other than α and β ankyrin and hence enter the axon as a component of axonal transport [57].

Are Other Components of the Erythroid Membrane-Skeleton Expressed in Neurons?

The demonstration that polypeptides structurally and functionally related to the erythroid membrane-skeleton proteins spectrin and ankyrin are expressed in neurons raises the possibility that further counterparts of erythroid-skeletal proteins may be present in these cells. Recently, two independent studies have addressed this question using antibodies raised against erythrocyte protein 4.1 with different results. Studies from this laboratory [34] did not detect immunoreactive forms of this protein in chicken cerebellum or retina by immunofluorescence or immunoautoradiography, although structurally similar proteins were unambiguously detected in both avian and mammalian erythrocytes and lens fiber cells. On the other hand, Goodman et al [32] have reported that a polypeptide serologically related to mammalian erythrocyte protein 4.1 is present in porcine cerebellum with a distribution similar to that of brain ($\alpha\gamma$) spectrin. At present, the reason(s) for these differences is not known and requires further investigation.

CONCLUSIONS

Our ongoing studies, reviewed here, have sought to gain an insight into the molecular basis of neuronal morphogenesis and the role of the membrane-skeleton in the establishment and maintenance of the structural and physiological asymmetry of the neuron. Using the components of the erythrocyte membrane-skeleton as a model system, we have demonstrated an asymmetry in the molecular composition of the neuronal plasma membrane which appears to parallel the characteristic structural asymmetry of the mature neuron; α ankyrin and $\alpha\beta'$ spectrin are detected exclusively in the perikarya and dendrites, whereas β ankyrin and $\alpha\gamma$ spectrin are present throughout the neuron. Furthermore, by analyzing the sequence of molecular events involved in the establishment of this asymmetry in the membrane-skeleton during neuronal morphogenesis, we have identified two discrete patterns of spectrin and

ankyrin isoform expression and assembly on the plasma membrane during the phases of cell growth and terminal differentiation. When superimposed upon one another in the mature neuron, these two classes of spectrin and ankyrin isoforms may be responsible for establishing the structural asymmetry of the membrane-skeleton. At present, the details of the molecular mechanism(s) involved in maintaining this asymmetry in the membrane-skeleton are unknown. However, our analysis of the temporal and spatial accumulation in neurons of ankyrin and spectrin isoforms has demonstrated that this asymmetry is maintained actively in adult neurons. Furthermore, they have identified one of the ankyrin isoforms, α ankyrin, as a candidate for the membrane attachment site of $\alpha\beta'$ spectrin in the perikarya and dendrites, and hence the potential molecular basis for the active segregation of the two spectrin forms. This hypothesis is consistent with all the known biochemical properties of ankyrin in erythrocytes and neurons, and the kinetics of assembly of spectrin onto the plasma membrane of erythrocytes. However, conclusive evidence for the role of the various ankyrin isoforms in mediating the segregation of the two spectrin isoforms must await the purification of the individual polypeptides and their subsequent reconstitution in a well-defined in vitro system [52].

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