changed further upon ligand binding.

Our work, together with earlier observations on cytochromes c, c′, and c_3, suggests a possible role for heme orientation in regulating electron transfer rates in biological systems. The results of these spectroscopic studies show that the heme groups retain their mutually perpendicular orientation in both oxidized and reduced forms. Intramolecular electron transfer of heme c to heme d_1 in cytochrome cd_1 is relatively sluggish in the absence of dioxygen ($k \simeq 0.3$ sec^{-1} at 25°C) (*12, 15*), in contrast to rates observed for kinetically facile complexes of cytochromes. Since the average interheme distance in cytochrome cd_1 (*12, 15*) is comparable to that in kinetically facile multiheme oxidation-reduction enzymes (*2–11*), the perpendicular disposition of the heme c and heme d_1 groups is logically responsible for the slow rate of electron transfer.

We have previously shown that the orbitals associated with porphyrin-to-metal and metal-to-porphyrin charge-transfer transitions are identical to those involved in oxidation-reduction reactions (*20, 26*). In general, the probability of charge-transfer transitions polarized perpendicularly to the heme plane is small in comparison to that for charge-transfer transitions polarized in the heme plane (*20*). In oxidation-reduction reactions the probability of the electron transfer event is determined by the extent of orbital overlap between the donor and acceptor molecules according to a quantum mechanical description (*5, 6*) that is analogous to that for electronic transitions to excited states. Thus, the perpendicular orientation of the heme c and heme d_1 groups in cytochrome cd_1 may also predispose to a small transition probability (that is, small rate constant) for the redox process because of similar disposition of the orbitals of the porphyrin ring and the iron involved in the oxidation-reduction process.

The physiological function of cytochrome c_3 with four hemes arranged in approximately perpendicular pairs appears to be the storage of reducing equivalents (*4*); similarly, on the basis of kinetic studies of electron transfer reactions of cytochrome cd_1 (*12, 15*), there is now a strong hint that the ability of this multiheme enzyme to store electrons prior to intramolecular reduction steps may be enhanced by a perpendicular heme-heme disposition. In contrast, cytochrome complexes characterized by kinetically facile intermolecular electron transfer appear to have mutually parallel heme orientations (*3, 7–11*). This correlation suggests that electron transfer rates in multiheme enzyme complexes are strong functions of both orientation and distance and that a perpendicular heme-heme orientation may be an important factor in specifying kinetically slow steps in a sequential series of electron transfer reactions.

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Erythrocyte Form of Spectrin in Cerebellum: Appearance at a Specific Stage in the Terminal Differentiation of Neurons

Abstract. *The developing chicken cerebellum contains two forms of the plasma membrane–associated actin-binding protein spectrin. The brain form, αγ-spectrin (fodrin), is expressed constitutively in all neuronal cell bodies and processes during all stages of cerebellar morphogenesis. On the other hand, the erythrocyte form, αβ′β-spectrin, accumulates exclusively at the plasma membrane of the cell bodies of Purkinje and granule cells and of neurons in cerebellar nuclei, but only after these cells have become postmitotic and have completed their migration to their final positions in the cerebellum. The appearance of αβ′β-spectrin coincides temporally with the establishment of axosomatic contacts on these three neuronal cell types, which suggests that αβ′β-spectrin accumulates in response to the formation of functional synaptic connections during cerebellar ontogeny.*

The morphogenesis of the major neuronal cell types of the cerebellum has been extensively studied by means of light and electron microscopy (*1*). In general, cells undergo an initial proliferative phase, and then a postmitotic phase during which each cell type migrates to a specific area of the cortex. Once the cell reaches its designated position, it undergoes terminal differentiation upon the establishment of synaptic connections with other neurons. Thus, the cerebellum provides an ideal system to study the development of the chemical and structural heterogeneity of synapses during neuronal morphogenesis.

Recently, we investigated the distribution in adult chicken cerebellum (*2*) of an actin-binding protein that is antigenically related, and functionally analogous, to erythrocyte spectrin (*3–7*). We showed that there are two forms of spectrin in the adult cerebellum (*2*). The predominant form is αγ-spectrin [brain form, referred to previously as fodrin (*3*)], which is present throughout all axonal processes and dendritic arborizations in the three cortical layers of the cerebellum, and in the white matter. The minor form is αβ′β-spectrin [erythrocyte form, also present in striated muscle (*7, 8*)], which is found exclusively in Purkinje and granule cells and the neurons in the cerebellar nuclei, where it is confined to the plasma membrane of the cell bodies and the initial segment of the dendritic trunks emanating therefrom. These results indicate that there is an anisotropy in the molecular composition of the membrane-cytoskeleton of these fully differentiated neurons (*2*). We have now analyzed by indirect immunofluorescence microscopy, the temporal expression of the two classes of cerebellar spectrin during development of the chick cerebellum in order to determine whether the establishment of this anisotropy is correlated with the onset of synaptogenesis and terminal differentiation of these neurons.

Immunofluorescence with antibodies to erythrocyte α-spectrin reveals that brain α-spectrin is present in all the developing layers of the cerebellum [Fig. 1E shows a representative section through the various layers of the cerebellum from a 19-day-old chicken embryo (embryonic day 19)]. Since we have shown previously that equimolar amounts of α- and γ-spectrin coimmunoprecipitate as a complex from cerebellum (*2, 7*), we assume that the distribution of γ-spectrin is identical to that shown for α-spectrin.

In striking contrast to α-spectrin, immunofluorescence with antibodies to erythrocyte β-spectrin reveals that, throughout all stages of cerebellum morphogenesis, the cells of the external granular layer and the presumptive postmitotic granule cells that are migrating through the molecular layer and through the Purkinje cell layer exhibit no fluorescence (Fig. 1, B to D). The numerous developing dendritic processes of Purkinje cells and the parallel fibers of granule cells also exhibit no fluorescence, as has been shown in adult cerebellum (*2*). 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 17 and which are forming the internal granular layer (not shown). The first indication of β′β-spectrin accumulation is at embryonic day 17 when small foci of β-spectrin fluorescence can be detected at the plasma membrane of cell bodies of granule cells below the Purkinje cell layer (Fig. 1B). The degree of this fluorescence becomes more prominent by embryonic day 19 (Fig. 1C), and by hatching (day 21) is essentially indistinguishable from that observed in the adult cerebellum (Fig. 1D). Before the onset of granule cell migration at about embryonic day 15, the Purkinje cells have already migrated and lined up to form a distinct layer. However, β′β-spectrin is not detectable in the cell bodies of these cells until embryonic day 16–17 and approximately 24 hours before it is detected in the granule cells (Fig. 1B).

In general, axonal processes in the white matter exhibit no fluorescence with antibodies to β-spectrin during all stages of cerebellar morphogenesis (Fig. 2B). A notable exception are the cell

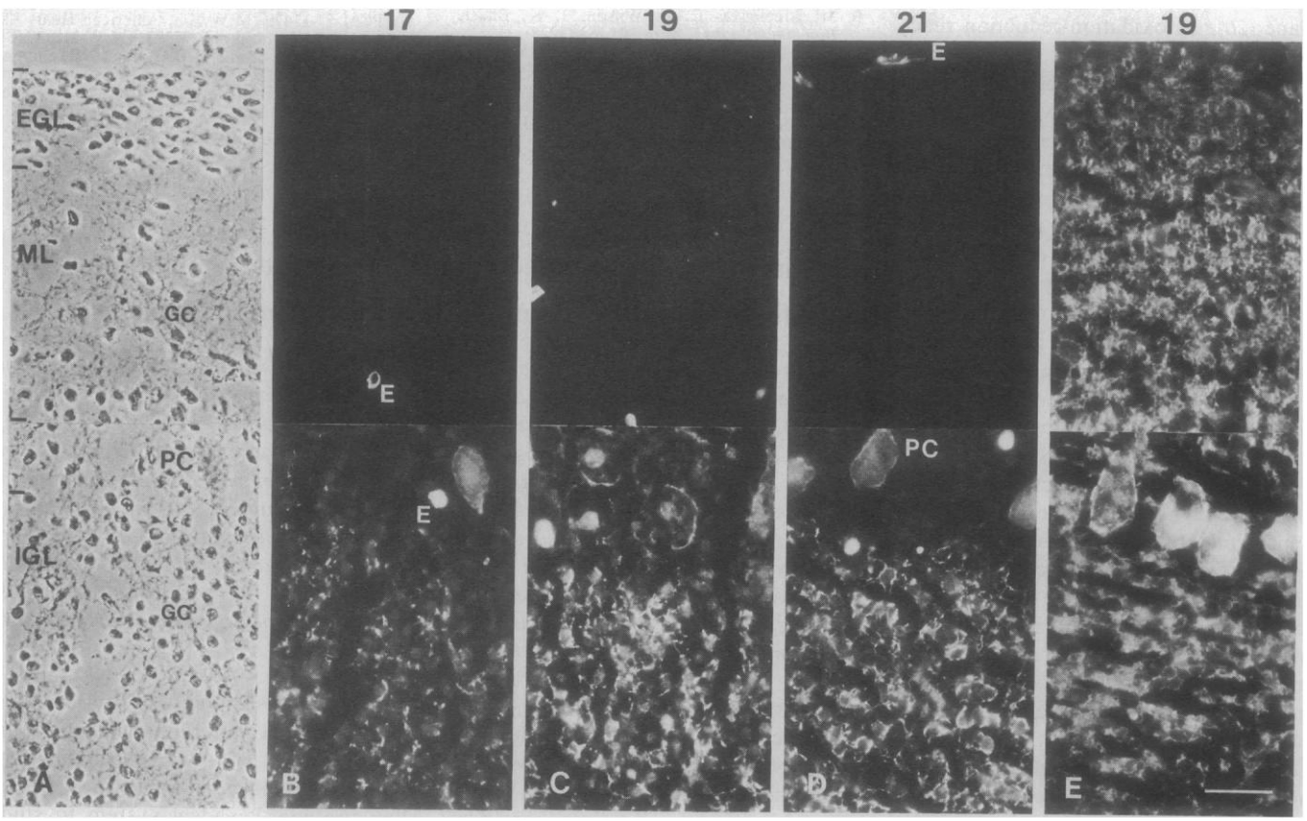

Fig. 1. Indirect immunofluorescence performed on frozen sections of cerebellum (*2, 7*) with erythrocyte α-spectrin (E) or β-spectrin (B–D) specific antibodies. (A) is the corresponding phase-contrast image of (B). Bar, 24 μm; *EGL*, external granular layer; *ML*, molecular layer; *PC*, Purkinje cell; *IGL*, internal granular layer; *GC*, granule cells; and *E*, erythrocytes. The numbers at the top indicate the day of embryonic development.

bodies of neurons in the cerebellar nuclei which, by embryonic day 19, exhibit β-spectrin-specific fluorescence in association with their plasma membrane (Fig. 2A), the intensity of which is indistinguishable from that in the adult.

Since it has been established that all migrating Purkinje and granule cells, and those that have reached their designated area in the cerebellum, are postmitotic (*1, 9*), we can conclude that the accumulation of β′β-spectrin is a postmitotic event and coincides with the phase of terminal differentiation of these cells and, presumably, of the neurons in cerebellar nuclei. The expression of αγ-spectrin, on the other hand, is constitutive throughout development in both mitotic and postmitotic cells. In this respect it is interesting that the accumulation of β′β-spectrin during skeletal muscle differentiation in vitro occurs also as these cells become postmitotic and enter their terminal phase of differentiation (*9*). Thus, the postmitotic accumulation of β′β-spectrin in terminally differentiating muscle cells and cerebellar neurons may be regulated by a morphogenetic molecular event common to these two topologically and functionally distinct cell types.

The temporal and spatial appearance of β′β-spectrin in Purkinje and granule cells and in neurons of cerebellar nuclei coincides remarkably with synaptogenetic events that take place on these three cell types at this stage of development (*1, 10, 11*). At about embryonic day 16–17, incoming climbing fibers first establish contact with Purkinje cell bodies (axosomatic contacts) before migrating and establishing contact with the dendrites of these cells within the molecular layer; an event which occurs well before the formation of axosomatic contacts between Basket cells and Purkinje cells. Similarly, incoming mossy fibers first establish contact with the cell bodies of granule cells at about embryonic day 17–18, before proceeding to form contacts with their developing dendrites and the establishment of initial glomeruli (*10*). The onset of β′β-spectrin accumulation coincides also with the appearance and distribution of synapsin I, a synaptic vesicle phosphoprotein whose appearance in the developing cerebellum parallels that of synapses (*12*), and of neuronal enolase (*13*), the glycoprotein thy-1 (*14*), and a 23,000-dalton neuronal mitochondrial-specific protein (*15*), all of which have been shown to appear in conjunction with the terminal differentiation of neurons. However, unlike these other protein markers, which are found throughout the central and peripheral nervous system, the accumulation of β′β-spectrin is specific to terminally differentiating cerebellar neurons (*2, 7*).

Thus β′β-spectrin appears to be a specific postsynaptic marker for the establishment of functional synapses of Purkinje and granule cell bodies during cerebellum morphogenesis. In addition, β′β-spectrin appears at the same time in the cell bodies of neurons in cerebellar nuclei onto which the axons of Purkinje cells and climbing fibers synapse, but it is absent from the processes in the molecular layer. Therefore, β′β-spectrin may accumulate specifically at the postsynaptic area of only the two major input and one major output relay synapses of the cortex of the cerebellum, regardless of whether the synapses are inhibitory or excitatory.

The constitutive expression of αγ-spectrin in all layers of the cerebellum, and the specific pattern of accumulation of αβ′β-spectrin in the cell bodies of terminally differentiating Purkinje and granule cells and of neurons in cerebellar nuclei, explains when the anisotropy in the distribution of αγ- and αβ′β-spectrin observed in adult cerebellum is established during cerebellar morphogenesis. However, the mechanism by which αβ′β-spectrin associates exclusively with the plasma membrane of the cell bodies and not the axons of these types of cerebellar neurons is at present unknown. One hypothesis is that there is a membrane receptor (or receptors) specific for β′β-spectrin, which is expressed in these three types of terminally differentiating neurons in response to the establishment of axosomatic contacts onto their cell bodies. The insertion of the receptor into the plasma membrane of the neuronal cell bodies would drive the stable assembly, and hence accumulation, of αβ′β-spectrin in these loci despite the presence of an excess of αγ-spectrin. This hypothesis of membrane receptor–driven spectrin subunit assembly and segregation is analogous to that shown recently to occur during the assembly of αβ′β-spectrin in erythroid cells during chicken embryo development (*16*). Identification of this receptor would help to elucidate the mechanism involved in the accumulation of αβ′β-spectrin in cerebellar neuronal cell bodies in conjunction with synaptogenesis which results in the establishment of the anisotropy in the molecular composition of the membrane-cytoskeleton.

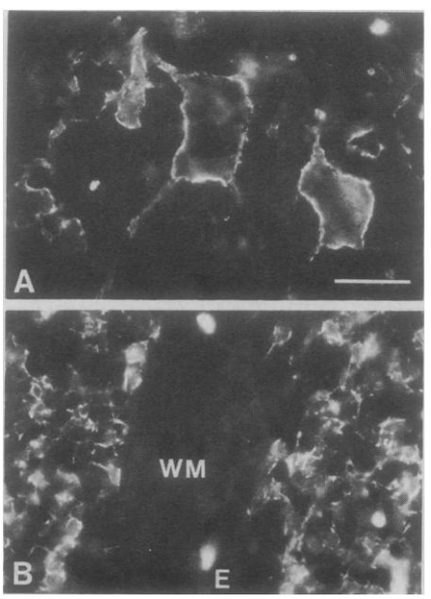

Fig. 2. Localization of β′β-spectrin at the plasma membrane of the cell bodies of neurons in cerebellar nuclei. Immunofluorescence with erythrocyte β-spectrin–specific antibodies on frozen sections of cerebellum from a 19- to 20-day-old chicken embryo (Fig. 1). (A) Section of white matter rich in cell bodies of neurons in cerebellar nuclei. (B) An adjacent section of the white matter flanked by the granular layer. *WM*, white matter; bar, 24 μm.

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