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Postmitotic Expression of Ankyrin$_R$ and β_R-Spectrin in Discrete Neuronal Populations of the Rat Brain

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Isoforms of ankyrin (ankyrin$_R$) are expressed in both the erythrocyte and the brain. Four cDNAs representing regulatory domains of ankyrin$_R$ expressed in the rat spleen and brain were cloned and sequenced. These different cDNAs were found to result from tissue-specific alternative mRNA processing of the ankyrin$_R$ regulatory domain. One of the isolated cDNAs was used to develop an antibody to brain isoforms of ankyrin$_R$, and this antibody was used to study the localization of ankyrin$_R$ in the rat brain. The protein was found to be widely expressed in neurons of the metencephalon but limited to a discrete subset of neurons in the rat forebrain. In the thalamus and areas of the basal ganglia, these neurons were grouped in defined nuclei, whereas in the cortex, hippocampus, and caudate putamen they appeared as isolated cells distributed randomly throughout these structures. A similar study using an antibody raised against erythrocyte spectrin (β_R) showed a comparable localization to that of ankyrin$_R$. Both proteins were expressed late in the developing rat brain, as part of the maturation stage of neural development. These data suggest a specific role for these erythrocyte structural proteins in the postmitotic development of a subset of neurons in the rat brain.

[Key words: ankyrin, spectrin, membrane skeleton, tissue-specific splicing, developmental expression, neuronal populations, cell-specific expression]

The spectrin-based erythrocyte membrane skeleton is a network of filamentous proteins that forms a two-dimensional lattice on the cytoplasmic surface of the erythrocyte plasma membrane (reviewed by Bennett, 1990; Palek and Lambert, 1990). The skeleton provides support and elastic maintenance of the erythrocyte shape during its passage through the vascular system, as well as restricting the lateral diffusion of integral proteins such as the anion channel (Tsuji et al., 1988). The membrane skeleton is linked to the lipid bilayer through the associations of a largely globular protein termed ankyrin, which interacts with both the β-spectrin subunit and the anion channel.

Isoforms of erythrocyte membrane skeleton proteins have been observed in a diverse range of tissues and organisms, where they have often been localized to specialized domains of the plasma membrane, such as areas of receptor capping in lymphocytes (Repasky et al., 1984) or the node of Ranvier in myelinated axons (Kordeli et al., 1990; Kordeli and Bennett, 1991). These observations suggest a role for the membrane skeleton in the maintenance and/or formation of specialized domains, presumably by restricting the lateral diffusion of specific integral proteins to these regions of the plasma membrane. This function would require that the ankyrin molecule serve as an adaptor between the membrane skeleton and a variety of integral proteins. Characterization of the ankyrin structure has revealed it to be uniquely suited for such a function.

Ankyrin$_R$ is the product of the ANK-1 gene locus on 8p11 (Lux et al., 1990b) and was first isolated from the human erythrocyte. It consists of three basic functional domains (Bennett, 1992). The membrane binding domain, which is involved in a variety of integral protein interactions (Davis et al., 1991; Srinivasan et al., 1992), consists of 22 copies of a 33 amino acid repeat motif (Lambert et al., 1990; Lux et al., 1990a) that has also been observed in a wide variety of proteins ranging from mammalian transcription factors to α-latrotoxin of the black widow spider (Michaely and Bennett, 1992). This 33 amino acid motif is believed to play a crucial role in protein–protein interactions (Davis et al., 1991; Henkel et al., 1992). A second major domain of ankyrin$_R$ is the spectrin binding domain, which contains the major site for association with the β-subunit of erythrocyte spectrin within its N-terminus (Davis and Bennett, 1990). The third domain of the molecule, termed the regulatory domain, is a major site for alternative mRNA processing in the human erythrocyte and appears to affect the interactions of both the membrane and spectrin binding domains (Hall and Bennett, 1987; Davis et al., 1992).

A wide range of ankyrin functional diversity is achieved through the use of multiple genes and/or alternative mRNA processing. Members of the ankyrin gene family are highly homologous in their membrane and spectrin binding domains but are divergent in their regulatory domains (Otto et al., 1991). Three different ankyrin genes are known to be expressed in the rat brain. Ankyrin$_B$ is a product of the ANK-2 locus on 4q25-q27 (Tse et al., 1991) and is the major ankyrin isoform in the brain. Ankyrin$_B$ is expressed by neuronal and glial cells (Kordeli et al., 1990; Kunimoto et al., 1991; Otto et al., 1991). Ankyrin$_{NODE}$ is as yet uncharacterized but appears to be a unique gene product localized to the initial segment and node of Ranvier of myelinated axons (Kordeli and Bennett, 1991). Ankyrin$_R$ is also expressed in the rat CNS. This conclusion results from immunofluorescent data obtained using an antibody against an alternatively processed exon of the human ANK-1 gene sequence and from mutant mice (the nb, or normoblastosis, mutation) with ankyrin$_R$ deficiency resulting in both hematological

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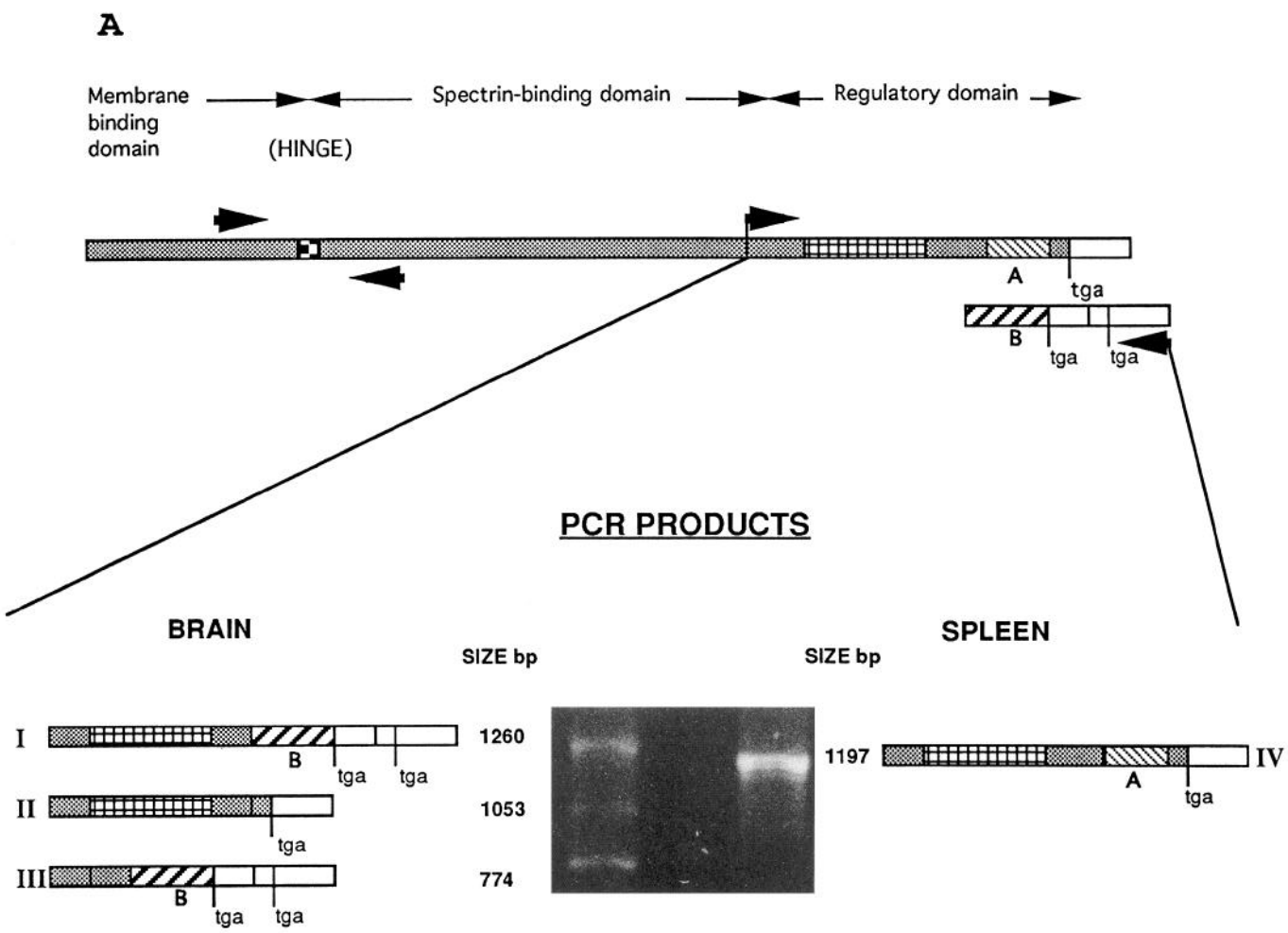

B

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EIVNMLEGSGRQSRNLKPDRRHgDReYSLSPSQvNGYSSLQDELLSPASL   50
hyALpSPLcADQYWNEVAviDAIPLAATEHDTMLEMSDMQVWSsGLTPSL  100
VTAEDSSLECSKAEDSDAipewkLEGAhSEdsqGPELGSqdLVEDDTVDS  150
DATNGLaDLLqQEEGQRSEkKRQevsGteQDtEtEVSLVSGqQRvhARIT  200
dSPsVrQVldRSQaRtvDWykqGSaavhpQeAtQsSWQEEVTQGPqSFQr  250
riTtiqGpEPGalQEYEqVvVStrEHvqrgpPEsdtpkAgkDpslwapes  300
aFsQeVQGdElQNIPGEQVTEEQFTDEQGNIVTKKIIRKVVRQvDsSgAi  350
dtQqHEEViVEGPLaDPgdLEVDIesFMKltKDHTSTPkP* (A)        390
dtQqHEEVELRGSGLQPDLIEGRKGAQIVKRASLKRGKQ* (B)         389
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C

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DEILDVSEDEGTAHISLTGdELvgsKAE   28
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Figure 1. RT-PCR of ankyrin$_R$ regulatory domain from rat brain and spleen mRNA. *A,* Linear diagram of ankyrin$_R$ showing the relative position of PCR primers (►) and areas of alternative mRNA processing in the hinge and regulatory domain. Alternate C-termini [either acidic (*A*) or basic (*B*)] for ankyrin$_R$ are shown (Lambert et al., 1990; Lux et al., 1990a). Also shown is an ethidium bromide–stained 1.2% agarose gel of DNA products obtained after RT-PCR of mRNA from either the rat brain or spleen. Primers used flanked the regulatory domain of the human ankyrin$_R$ sequence (see Materials and Methods). Diagram illustrates the composition of alternatively processed mRNA products. Areas of coding sequence are shown by ▨. *Open boxes* represent areas of untranslated sequence. ▨, ▨, ▨, and ▨ are regions of the molecule subject to alternative mRNA processing. *tga* represents a stop codon. *B,* The derived amino acid sequence of the rat ankyrin$_R$ regulatory domain, with areas of alternative mRNA processing *underlined.* The area shown is equivalent to residues 1477–1879 of the human cDNA ankyrin$_R$ sequence (Lambert et al., 1990). *Boldface uppercase letters* represent amino acids identical between human and rat. The alternate basic C-terminus is shown (*B*). *C,* Derived amino acid sequence from the rat ankyrin$_R$ "hinge" region. The area shown is equivalent to residues 810–829 of the human ankyrin$_R$ cDNA sequence. *Boldface uppercase letters* represent amino acids identical between human and rat. An insertion observed in mRNA from the rat brain is *underlined.*

and neurological abnormalities (Kordeli and Bennett, 1991; Peters et al., 1991). These data suggested a limited expression of ankyrin$_R$ in the rat CNS. Although the function of ankyrin$_R$ in the erythrocyte is well established, the function of this molecule in the brain is unknown with the exception that deficiency of ankyrin$_R$ appears to result in degeneration of Purkinje cell neurons in the *nb* mutation.

In this study we gained further insight into the function of ankyrin$_R$ in the brain by studying its expression in the rat CNS. We cloned four cDNAs encoding the ankyrin$_R$ regulatory do-

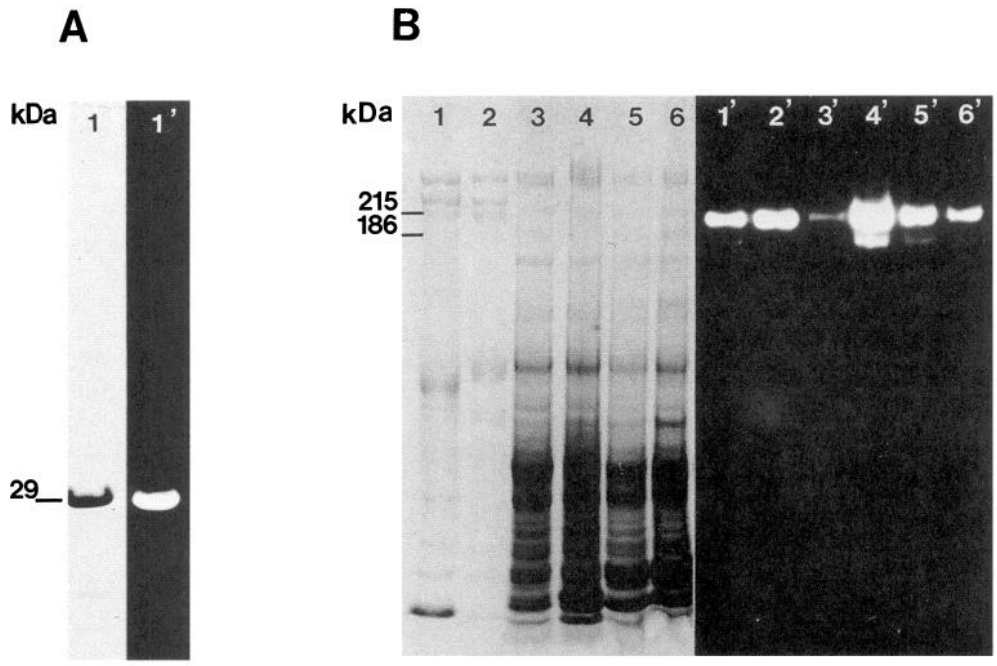

Figure 2. Preparation and characterization of an antibody to the rat ankyrin$_R$ regulatory domain. *A*, Coomassie blue–stained (*lane 1*) separation of recombinant protein RDIII representing an ankyrin$_R$ regulatory domain and the corresponding immunoblot (*lane 1'*) stained with an antibody to human erythrocyte ankyrin. *B*, Coomassie blue–stained separation (*lanes 1–6*) and corresponding immunoblot from SDS-PAGE separation (*lanes 1'–6'*) of human (*lane 1*) and rat (*lane 2*) erythrocyte membrane proteins. *Lanes 3, 4, 5* and *6* are brain membrane preparations from the adult rat forebrain, cerebellum, brainstem, and spinal cord, respectively.

main from the rat brain and spleen, representing products of tissue-specific alternative mRNA processing. One of these cDNAs was used to make an antibody against brain-specific isoforms of the ankyrin$_R$ regulatory domain. Immunofluorescence reveals that the expression of ankyrin$_R$ is limited to neurons of discrete nuclei in the rat thalamus and basal ganglia and to isolated neurons in the rat cortex and hippocampus. A second antibody specific to the β-subunit of red cell spectrin shows a similar restricted localization, suggesting the presence of a subset of neurons in the rat brain that characteristically express genes encoding proteins of the erythrocyte membrane skeleton. Developmental studies showed that these proteins are expressed late in neuronal development, during the final maturation stages of neuronal development.

Materials and Methods

Isolation of ankyrin$_R$ regulatory domain cDNA sequence from the rat brain and spleen. Reverse transcription and the polymerase chain reaction (RT-PCR) were used to isolate cDNA sequences corresponding to the regulatory domain of ankyrin$_R$ as expressed in the rat brain and spleen. Total RNA was isolated from the rat brain and spleen using RNAzol (Cinna Biotecx, Houston, TX) and further purified using PolyAT-tract (Promega Corp., Madison, WI) to give polyA$^+$ RNA. We used 1 μg of each mRNA in a 20 μl reverse transcription reaction containing 1 mM dNTPs, 20 mM dithiothreitol, 2U/μl RNasin (Promega), 200 U of MuLV-reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), and 10 pmol of a downstream primer representing nucleotides 5742–5763 of the human ankyrin$_R$ cDNA sequence. After incubation at 42°C for 1 hr, reactions were terminated by heating and used in a 100 μl PCR reaction in the presence of 30 pmol of upstream primer (nucleotides 4501–4523) and a final concentration of 30 pmol downstream primer. The relative position of PCR primers within the ankyrin$_R$ sequence is shown in Figure 1*A*. Reactions were carried out for 30 cycles using an annealing temperature of 50°C. Reaction products were separated by agarose gel electrophoresis and transferred to nitrocellulose filters using the method of Southern (1975). Filters were probed with a human cDNA encompassing the regulatory domain of ankyrin$_R$. Positive bands were subcloned into pBluescript (Stratagene, La Jolla, CA) and sequenced.

A similar procedure was used to look for alternative mRNA processing of ankyrin$_R$ in a region of the molecule between the membrane and spectrin binding domains (the "hinge" region). In this experiment the downstream PCR primer consisted of nucleotides 2808–2829, and the upstream primer, of nucleotides 2296–2317 of the human cDNA sequence (Fig. 1*A*).

Preparation of antibodies. Regulatory domain product III, a 774 base pair (bp) PCR product from the rat brain (Fig. 1*A*), was expressed in bacteria and used to produce antibodies to brain isoforms of rat ankyrin$_R$. PCR was used to introduce an *Nhe* I site at the 5' end of the cDNA, allowing it to be subcloned into the pGemex (Promega) expression vector, with the accompanying deletion of the viral gene 10 protein normally present in fusion proteins produced by this vector. This modification resulted in the presence of only three foreign amino acids at the amino terminus of the expressed protein (Davis et al., 1991). The recombinant plasmid was then transformed into the *Escherichia coli* host strain BL21 (DE3) pLysS (Studier et al., 1990), which resulted in low basal expression and high levels of inducible expression of the recombinant protein. Low basal expression proved advantageous, as the recombinant ankyrin$_R$ regulatory domain was found to inhibit bacterial growth severely.

Recombinant proteins were precipitated from bacterial cell lysates and purified using gel filtration on Superose 12 (Pharmacia LKB Biotechnology, Piscataway, NJ) and ion exchange chromatography on a Mono-S column (Pharmacia LKB Biotechnology). Purification of the product III recombinant was followed by SDS-PAGE and immunoblotting using an antibody to human erythrocyte ankyrin$_R$ that showed cross-reactivity to the recombinant protein (Fig. 2*A*). The recombinant protein was finally purified by SDS-PAGE and the gel slice injected into rabbits. Antibodies to recombinant product III were affinity purified against recombinant product III immobilized on Sepharose CL-6B.

The preparation and characterization of an antibody to the erythrocyte β-spectrin subunit have previously been described (Hu et al., 1992). Erythrocyte β-spectrin (β_R) obtained by preparative SDS-PAGE of erythrocyte membranes was used as an immunogen and the sera were adsorbed against immobilized brain spectrin to deplete cross-reactivity with the brain spectrin subunit (β_G). The remaining serum was then affinity purified against erythrocyte spectrin. This affinity predominantly recognized a 270 kDa polypeptide in the rat brain representing the product of an alternatively processed form of the β_R-spectrin mRNA present in both brain and skeletal muscle (Winkelmann et al., 1990). This antibody showed a weak cross-reactivity with bovine brain spectrin and no cross-reactivity with α-spectrin.

Gel electrophoresis and immunoblot analysis. Sprague–Dawley rats of various ages as indicated in the text were anesthetized and perfused with 150 mM NaCl, 10 mM sodium phosphate, pH 7.5, 2 mM EDTA, 0.01% (v/v) diisopropyl fluorophosphate, pH 7.5, 5 μg/ml pepstatin,

and 5 μg/ml leupeptin. Brains were removed and dissected into forebrain, cerebellum, and brainstem. Samples for electrophoresis were prepared as previously described (Kunimoto et al., 1991) and fractionated by SDS-PAGE on 3.5–17% exponential gradient gels before immunoblotting. Antibodies were visualized using ^{125}I-labeled protein A and autoradiography (Davis and Bennett, 1983). Samples of rat and human erythrocyte membrane proteins were prepared as previously described (Bennett, 1983).

Immunocytochemical procedures. Rats of various ages representing the littermates of animals used for immunoblot analysis were perfused with 150 mM NaCl, 10 mM sodium phosphate, pH 7.5, and 50 U/ml heparin, followed by 2% paraformaldehyde in the same buffer. Whole brains were removed and fixed for a further 2 hr before cryoprotection in 25% sucrose and freezing in isopentane cooled by liquid nitrogen. Cryosections were cut at 4 and 10 μm and mounted on Vectabond-treated glass slides. Sections were incubated overnight at 4°C with primary antibody concentrations of 5 μg/ml. Sections were extensively washed in PBS containing 0.1% Triton X-100 before visualization by indirect immunofluorescence (Kordeli et al., 1990).

Results

Brain-specific isoforms of ankyrin$_R$ are generated by tissue-specific alternative mRNA processing

Cloned cDNAs encoding isoforms of the ankyrin$_R$ regulatory domain expressed in the rat brain and spleen were isolated using RT-PCR. Figure 1*A* shows PCR products from rat brain and spleen mRNA amplified using primers flanking the regulatory domain of human erythrocyte ankyrin$_R$ and separated on a 1.2% agarose gel. The identity of these products was confirmed using Southern hybridization with a human cDNA clone for ankyrin$_R$. The products were subsequently cloned and sequenced. The derived amino acid sequence of the rat ankyrin$_R$ regulatory domain is shown in Figure 1*B*. We cloned three cDNAs encoding the ankyrin$_R$ regulatory domain from the rat brain and one from the rat spleen, with differences in sequence being due to tissue-specific alternative mRNA processing (Fig. 1*B*).

Two regions of alternative mRNA processing were observed in the regulatory domain. The largest region consisted of a peptide of 162 residues (Fig. 1*B*) deleted in product III from the rat brain. This peptide had previously been shown to function as an allosteric inhibitor of ankyrin function (Davis et al., 1992). The second region was at the 3′ end of the coding region and allowed the protein to terminate in an acidic (product IV from the spleen) or basic (products I and III from the brain) stretch of residues, or neither of these (product II from the brain). These two regions of alternative processing have also been observed in the human cDNA sequence for ankyrin$_R$ as cloned from a reticulocyte cDNA library (Lambert et al., 1990; Lux et al., 1990a), suggesting that the tissue-specific nature of this alternative mRNA processing is a species-related phenomenon. The relative abundance of different alternatively processed mRNA transcripts encoding ankyrin$_R$ in the human reticulocyte has not been determined. Hence, "brain" transcripts such as those encoding the basic stretch of residues at the protein C-terminus might represent only a small fraction of human erythrocyte ankyrin$_R$ transcripts. Overall homologies between the rat and human ankyrin$_R$ regulatory domains was 72%, with stretches of highest homology in the areas of alternative mRNA processing, the basic C-terminus being 100% conserved (Fig. 1*B*).

Further alternative mRNA processing was also seen in the "hinge" region (Fig. 1*A*) where 24 base pairs were inserted after nucleotide 2670. This insertion was only found in mRNA from the rat brain and was not found in mRNA from the rat spleen. Figure 1*C* shows the derived amino acid sequence of this insertion.

Alternative "hinge" and regulatory domain sequences in cDNAs from the rat brain and spleen demonstrate that alternative mRNA processing of ankyrin$_R$ is tissue specific in rodents, particularly at the C-terminus, and results in a number of isoforms specific for the brain. The same mechanism is used to generate diversity in these brain isoforms by allowing a number of combinatorial possibilities between different regions of alternative mRNA processing.

The 774 bp RT-PCR product (product III) was expressed and used to produce an antibody specific to ankyrin$_R$ isoforms. This product was utilized because it contained the highest proportion of invariant coding sequence, thereby allowing the production of an antibody that would recognize all brain isoforms of ankyrin$_R$. This was in contrast to previous studies of ankyrin$_R$ expression that employed a peptide antibody raised against sequence from an alternatively processed exon of human ankyrin$_R$ and hence did not recognize all brain ankyrin$_R$ isoforms (Kordeli and Bennett, 1991). The pure recombinant protein used as an immunogen is shown in Figure 2*A*, and the characterization of the corresponding anti-ankyrin$_R$ antibody, in Figure 2*B*. The antibody recognizes the major 220 kDa isoform of ankyrin$_R$ in the rat brain (lanes 3–6) along with a polypeptide at 186 kDa that may represent one or both of the alternatively processed transcripts indicated in Figure 1*A*. The 186 kDa polypeptide is not present in SDS-PAGE separations of rat erythrocyte membrane proteins (lane 2), concurring with the results shown in Figure 1*A* and suggesting the presence of only one ankyrin$_R$ transcript in mRNA from the rat spleen. The 186 kDa peptide is also not present in SDS-PAGE preparations of bovine and porcine erythrocyte membrane proteins (V. Bennett, unpublished observations). Both the 220 kDa and 186 kDa protein isoforms are observed in preparations of human erythrocyte membrane proteins (Fig. 2*B*, lane 1), affirming species differences in the tissue-specific nature of this alternative mRNA processing and suggesting that alternative mRNA processing of ankyrin$_R$ in erythrocytes is specific to humans.

Ankyrin$_R$ is predominantly expressed in neurons of discrete nuclei

Antibodies raised against the recombinant ankyrin$_R$ regulatory domain were used to study the expression of ankyrin$_R$ in the rat brain. Ankyrin$_R$ expression was restricted to discrete populations of neuronal cells in which the protein was localized to the plasma membrane of cell bodies and dendrites.

In the rat forebrain, ankyrin$_R$ staining reflected two distinct patterns of expression. In the thalamus and basal ganglia, ankyrin$_R$ staining was localized to neurons within defined nuclei as is seen in Figure 3, *A*, *B*, *D*, and *F*. Ankyrin$_R$ expression was also seen in isolated cells of forebrain structures such as the hippocampus (Fig. 3*E*). Both of these two staining patterns were seen in the basal ganglia (Fig. 3*F*), where ankyrin$_R$ expression in the caudate putamen (cpu) was limited to a small number of cells, in contrast to the staining seen in the globus pallidus (gp).

In the metencephalon, ankyrin$_R$ appeared to be expressed by the majority of neurons. This finding was similar to those of previous studies carried out with antibodies that showed cross-reactivity with other members of the ankyrin gene family or that were raised against sequence from an alternatively processed exon of the human ankyrin$_R$ sequence and hence were not able to recognize all brain ankyrin$_R$ isoforms (Kordeli and Bennett, 1991). Ankyrin$_R$ staining was seen in neurons of all the major metencephalon nuclei, including the pontine (Fig. 3*C*)

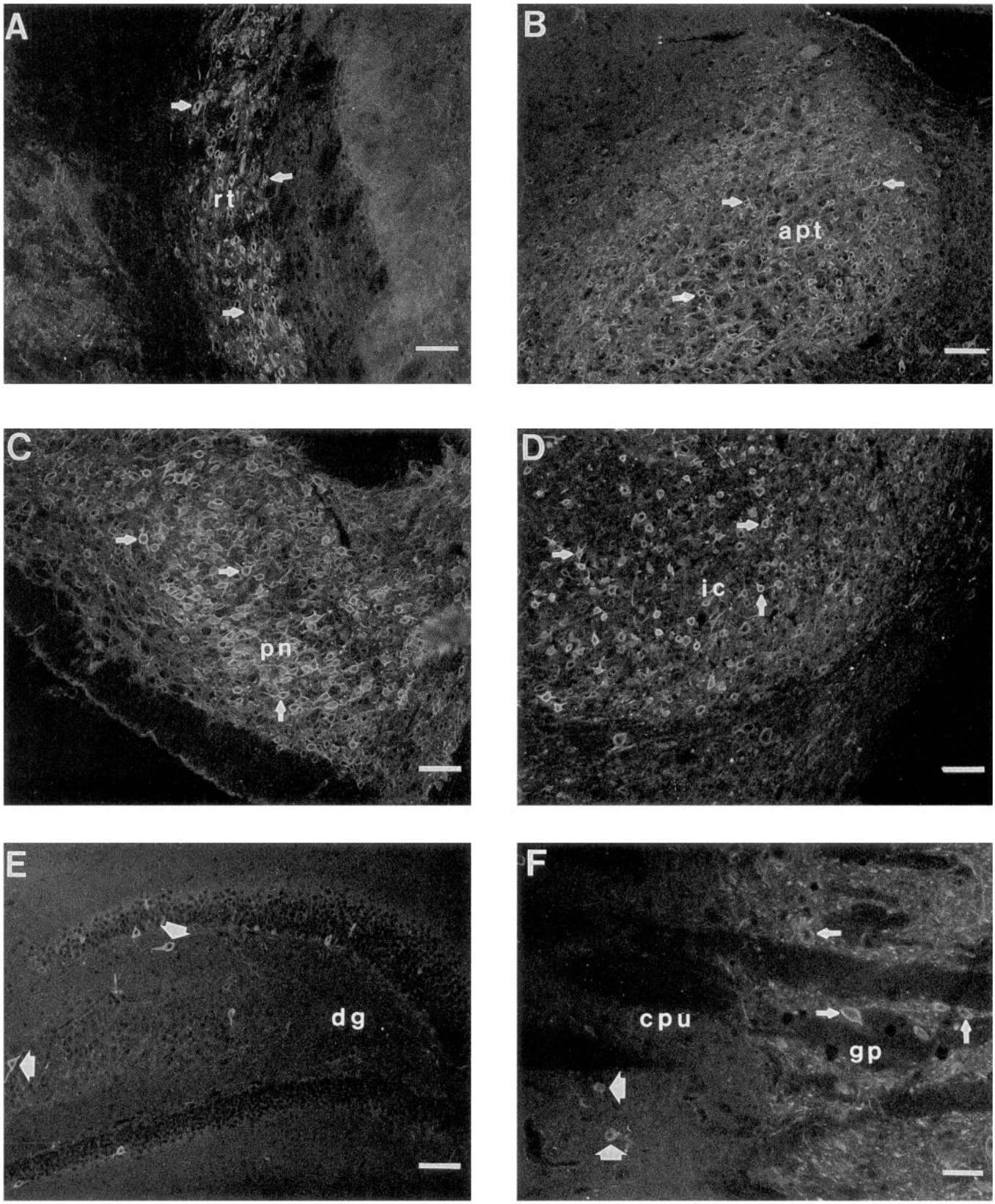

Figure 3. Immunofluorescence localization of ankyrin$_R$ in cryosections of the rat brain. Lateral 10 μm cryosections of the rat thalamus (*A*), midbrain (*B* and *D*), pons (*C*), hippocampus (*E*), and striatum (*F*) were stained with anti-ankyrin$_R$. Localized neuronal staining as indicated by *small arrows* was observed in the following nuclei: reticular thalamic (*rt*), anterior pretectal (*apt*), pontine (*pn*), inferior colliculus (*ic*), and globus pallidus (*gp*). The staining of isolated neurons (*large arrows*) was observed in the dentate gyrus (*dg*) area of the hippocampus as well as the caudate putamen (*cpu*). Scale bars, 100 μm.

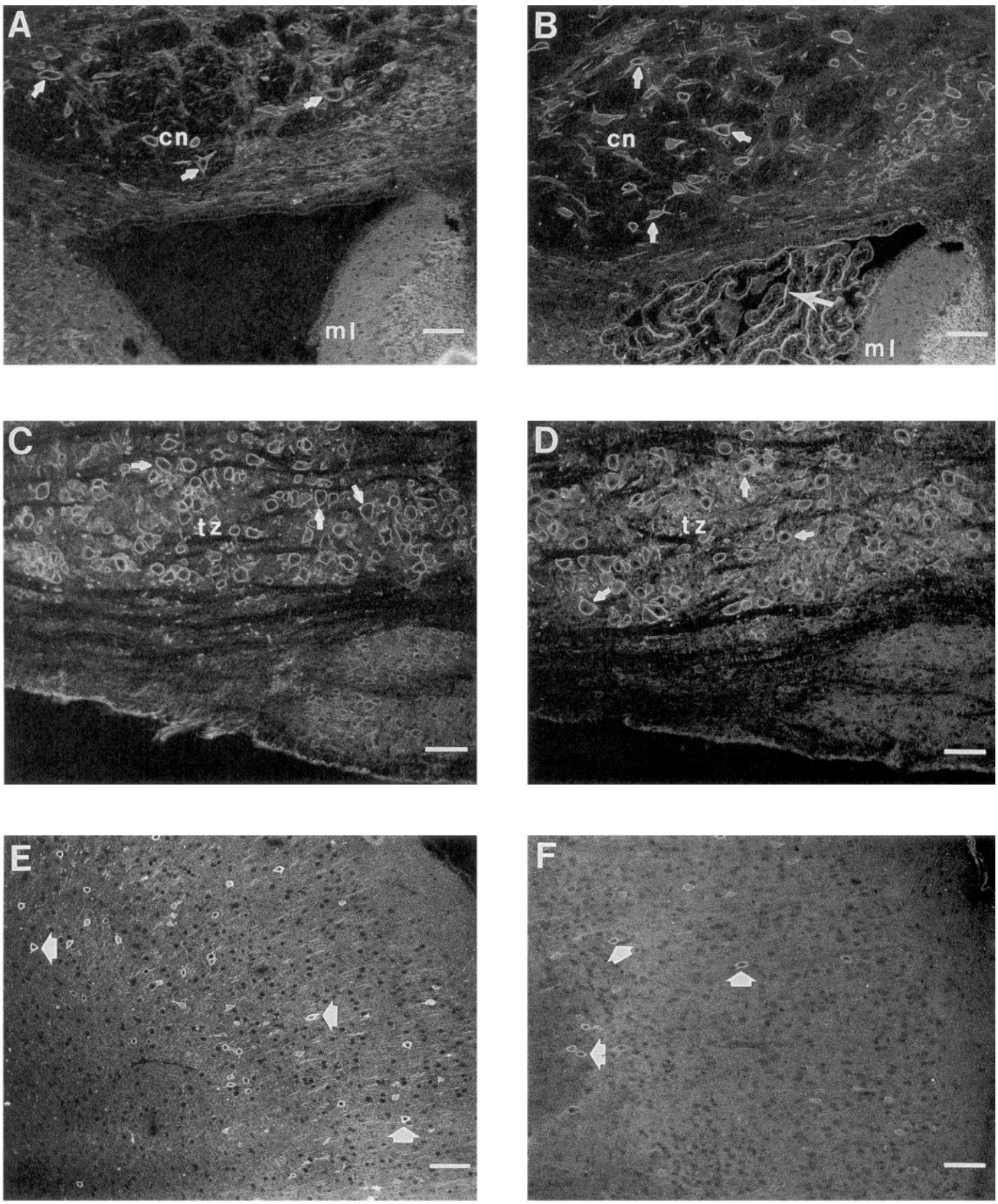

Figure 4. Immunofluorescence localization of ankyrin$_R$ and β_R-spectrin in the rat brain. Lateral 10 μm cryosections of the rat cerebellum (*A* and *B*) and occipital cortex (*E* and *F*), as well as coronal sections through the rat brainstem (*C* and *D*), were stained with either anti-ankyrin$_R$ (*A, C,* and *E*) or anti-β_R-spectrin (*B, D,* and *F*). *Small arrows* indicate staining of neurons in the cerebellar (*cn*) and trapezoid (*tz*) nuclei, and *large arrows,* staining of isolated neurons in the cortex. The molecular layer of the cerebellar cortex (*ml*) is marked for orientation. The *large arrow* in *B* indicates staining of epithelial cells. Scale bars, 100 μm.

and trapezoid (Fig. 4C) nuclei of the brainstem as well as neurons of the cerebellar cortex and large neurons of the cerebellar nuclei (Fig. 4A). The distribution of ankyrin$_R$ staining in the rat CNS is summarized in Table 1.

β_R spectrin has a pattern of expression similar to ankyrin$_R$ in the rat brain

Figure 4 shows comparisons of ankyrin$_R$ and β_R-spectrin staining in similar cryosections of the rat brain. In the metencephalon, ankyrin$_R$ and β_R-spectrin staining was seen in the same nuclei of the cerebellar cortex (Fig. 4A,B) as well as the brainstem (Fig. 4C,D). In the forebrain the β_R-spectrin staining was again similar to that of ankyrin$_R$, both antibodies recognizing neurons from the same nuclei as well as the isolated subsets of neurons in the hippocampus, cortex (Fig. 4E,F), and caudate putamen.

Immunofluorescence also showed the expression of ankyrin$_R$ and β_R-spectrin in the same cells using adjacent 4 μm cryosections through the interposed nuclei of the rat cerebellum (Fig. 5A,B).

Ankyrin$_R$ and β_R-spectrin are expressed late in neuronal development

Figure 6 shows the relative amounts of ankyrin$_R$ in three different regions of the rat brain during development. Quantitations of the relative amounts of ankyrin$_R$ were made from immunoblots of brain membrane samples stained with anti-ankyrin$_R$ (circles) and normalized for protein loadings as described in the caption. Figure 6 shows that amounts of ankyrin$_R$ were low in newborn rats and continued to rise in all areas of the brain during the first month of postnatal development. At 30 d after birth these levels still only approached 40–60% of those of the adult. β_R-spectrin levels as determined in the same fashion showed a similar pattern of expression as has previously been shown for this protein in total brain (Riederer et al., 1987). A comparison of β_R-spectrin expression (squares) with ankyrin$_R$ expression (circles) in the cerebellum is shown in Figure 6B and indicates a similar developmental pattern of expression. In the case of β_R-spectrin, however, this protein appeared to reach adult levels by 25 d after birth.

Table 1. Expression of ankyrin$_R$ in the rat brain

Region	Degree of expression
Forebrain	
Cerebral cortex	+
Olfactory bulb	+
Caudate putamen	+
Globus pallidus	++
Thalamus	++
Hippocampus	+
Midbrain	
Inferior colliculus	++
Superior colliculus	++
Hindbrain	
Pons	+++
Reticular formation	+++
Medulla oblongata	+++
Cerebellar nuclei	+++
Cerebellar cortex	+++

+, expression limited to isolated neurons; ++, expression in defined nuclei; +++, ubiquitous expression.

Ankyrin$_R$ and β_R-spectrin are expressed in postmitotic cells

Figure 7 shows cryosections of the developing rat cerebellum stained with anti-ankyrin$_R$. Figure 7A shows ankyrin$_R$ localization in the 10-d-old cerebellum. Antiankyrin$_R$ was shown to stain weakly the plasma membrane of Purkinje cells and isolated neurons in the granular cell layer. Staining of the Purkinje cell membrane was typically asymmetrical and localized to the area of the axonal hillock (Fig. 7A'). No staining of the cerebellar proliferative zone, representing undifferentiated mitotic neurons, was observed. A number of small cells were present in the molecular layer and may represent immature granule cells migrating from the inner proliferative layer to the granular cell layer (large arrows). These migrating cells were not stained by the ankyrin$_R$ antibody. By day 15 (Fig. 7B) staining around the cell body of Purkinje cells was strong and punctate staining of

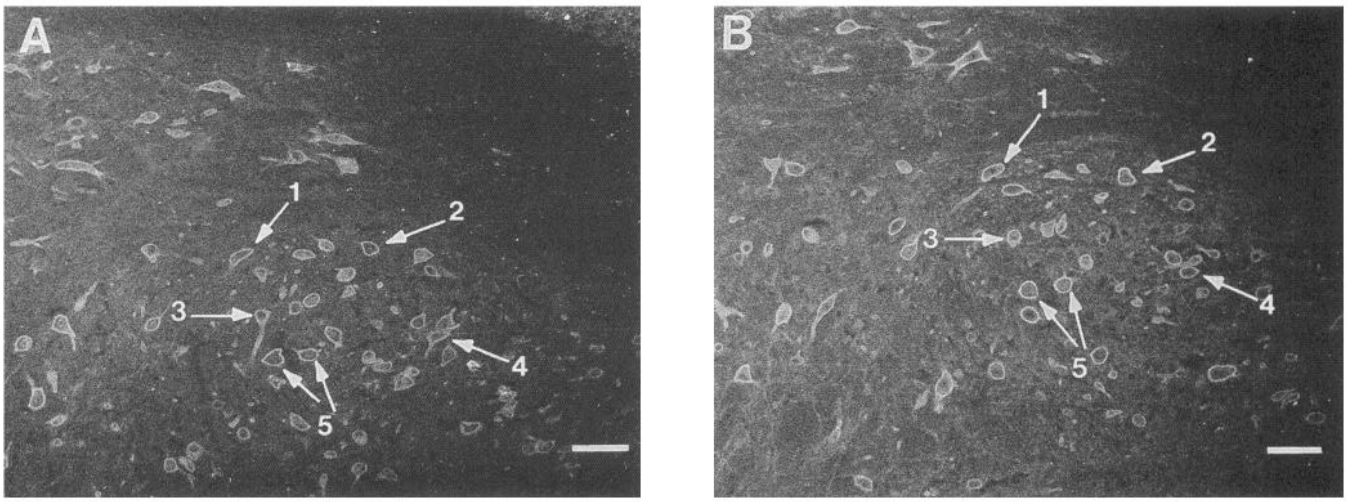

Figure 5. Immunofluorescence localization of ankyrin$_R$ and β_R-spectrin in adjacent sections of the rat cerebellum. Lateral 4 μm sections through the interposed nuclei of the cerebellum were stained with anti-ankyrin$_R$ (*A*) or anti-β_R-spectrin (*B*). Cells present in both sections are identified using *small arrows* and are *numbered identically* for comparison. Scale bars, 100 μm.

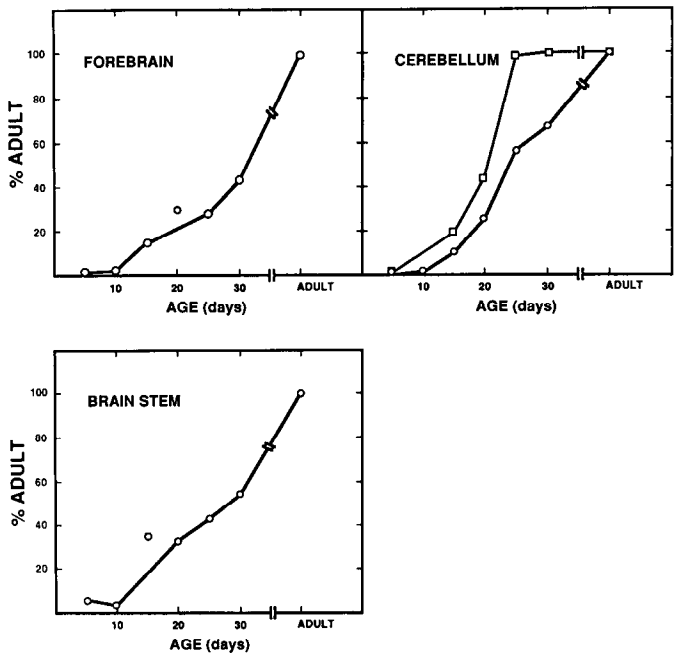

Figure 6. Quantitation of ankyrin$_R$ and β_R-spectrin in the developing rat brain. Protein samples from the rat forebrain, cerebellum, and brainstem at various days after birth were separated by SDS-PAGE as described in Materials and Methods. Immunoblots of these separations were carried out using either anti-ankyrin$_R$ (*circles*) or anti-β_R-spectrin (*squares*). Bound antibodies were visualized using ^{125}I-protein A and quantitated using densitometry of the corresponding autoradiograph. Identical separations stained with Coomassie blue were used for the normalization of protein loadings. Lanes were excised from the gel after extensive destaining and bound Coomassie blue was eluted from the gel using 25% pyridine (v/v) for 48 hr at room temperature. Relative protein loadings were determined by spectrophotometry at 610 nm and densitometric quantitations of ankyrin$_R$ and β_R-spectrin were adjusted accordingly. Quantitations shown are expressed relative to amounts observed in adult tissue (four animals of between 2 and 4 months of age) and are the mean of duplicate experiments.

the molecular layer was apparent in the orientation of the cerebellum shown. This punctate staining was reminiscent of the pattern observed for staining of dendritic spines at the light level (Kordeli and Bennett, 1991). These spines represent specialized protuberances from the dendritic shaft and in the Purkinje cell are the main sites of synaptic inputs from the parallel fibers of the cerebellar molecular layer. The identification of this punctate staining as dendritic spine labeling requires further confirmation, by either electron microscopy or double-labeling immunocytochemistry using a suitable marker for these structures. Staining was still absent from cells of the proliferative zone or cells undergoing apparent migration. Figure 7*C*, representing day 20, reflects the continuation of these trends, with abundant staining of both granular cells and Purkinje cells. Staining in the molecular layer was characteristic of the developing Purkinje cell dendritic tree. By day 30 (Fig. 7*D*) the staining pattern was similar to that of the adult.

β_R-spectrin expression in the developing rat cerebellum was similar to that of ankyrin$_R$. At postnatal day 10, weak β_R-spectrin staining was observed around the cell body of Purkinje cells and a few granule cells (Fig. 8*A*). Unlike ankyrin$_R$, the staining was punctate and appeared to be evenly distributed around the plasma membrane of the cells. No staining of cells in the proliferative zone was observed, or of cells undergoing migration to the granular cell layer (large arrows, Fig. 8*B*). The staining of mature Purkinje and granule cells continued to increase beyond postnatal day 15 (Fig. 8*B*), with the staining pattern at day 20 (Fig. 8*C*) similar to that of adult animals. In contrast to ankyrin$_R$, the β_R-spectrin antibody did not appear to stain the molecular layer of the cerebellum. This finding was in contrast to that of Riederer et al. (1987), who reported slight staining of the molecular layer when using an antibody to erythoid spectrin (240/235E). The absence of staining in the molecular layer may reflect a lack of antibody sensitivity or a peculiarity of Purkinje cells, as the dendritic processes of other cells, including those of the cerebellar nuclei (Fig. 4*B*), were clearly stained with this antibody.

Discussion

In this study we have isolated four cDNAs from rat brain and spleen mRNA encoding regulatory domains of ankyrin$_R$ produced by tissue-specific alternative mRNA processing. One of these cDNAs was then used to develop an antibody to brain isoforms of the ankyrin$_R$ regulatory domain. Immunofluorescence using the ankyrin$_R$ antibody, along with an antibody to β_R-spectrin, demonstrated the first example of a restricted expression for these two erythrocyte membrane proteins in the rat forebrain. In certain forebrain structures such as the hippocampus or cortex, expression of ankyrin$_R$ and β_R-spectrin was limited to a subset of isolated neurons distributed evenly throughout the structure. In the thalamus and basal ganglia, these two proteins were localized to neurons of defined nuclei. These results were in contrast to the almost ubiquitous expression of these molecules in neurons of the metencephalon. Proteins were localized to the plasma membrane of neuronal cell bodies and dendrites and no axonal staining could be seen. A study of the developmental expression of these proteins demonstrates that they were expressed late in the postmitotic phase of neuronal development.

The initial characterization of spectrin and ankyrin from the human erythrocyte suggested that these molecules behave as simple structural proteins providing support and maintenance of the erythrocyte shape. In the brain, however, the discovery of multiple gene products with restricted spatial and temporal expression indicates a more specialized function for these proteins. Developmental regulation of ankyrin and spectrin expression was first demonstrated for erythroid and nonerythroid isoforms in the developing avian brain (Nelson and Lazarides, 1984). Since then the differential expression of β_R- (erythroid) and β_G- (nonerythroid) spectrin and their segregation to different regions of neuronal cells have been described (Riederer et al., 1986, 1987; Siman et al., 1987), with β_R-spectrin segregating to the cell body and dendrites, and β_G-spectrin, to the axon and presynaptic terminal. In this study we extend these observations using isoform-specific reagents to propose that these proteins also show segregation at the regional and cellular level, with β_R-spectrin and ankyrin$_R$ expression limited to a subset of specialized neurons in the rat forebrain.

Alternative mRNA processing of the ankyrin$_R$ regulatory domain (shown in this study to be tissue specific) has been demonstrated to affect interactions of both the membrane and spectrin binding domains by acting allosterically at a site separate from the anion exchanger binding site in the membrane binding domain (Davis et al., 1992). It is of interest that a region of ankyrin$_R$ between the membrane and spectrin binding domains also shows evidence of tissue-specific alternative mRNA pro-

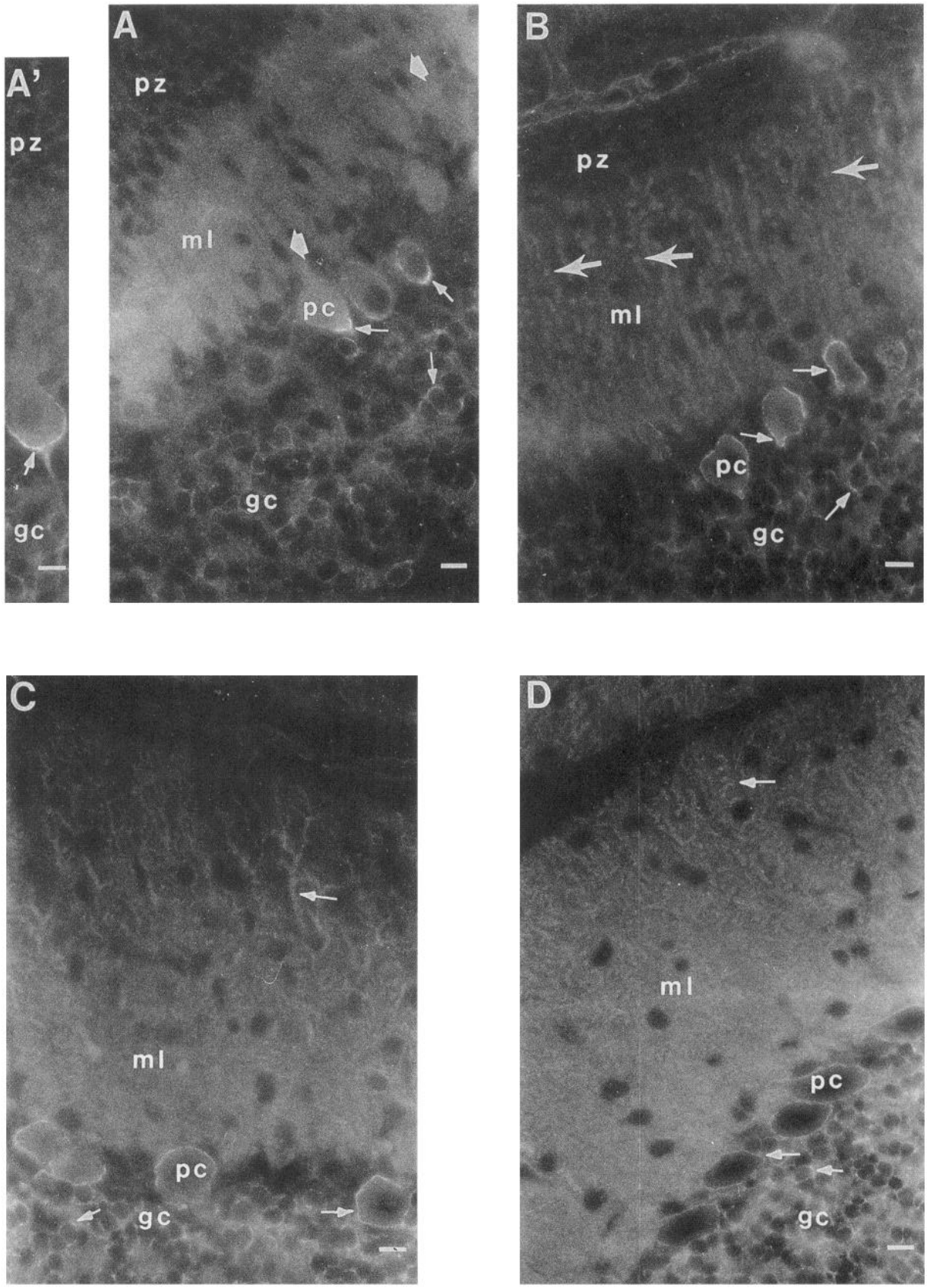

Figure 7. Immunofluorescent localization of ankyrin$_R$ in the developing rat cerebellum: lateral 4 μm cryosections of the developing rat cerebellum stained with anti-ankyrin$_R$. Micrographs represent postnatal day 10 (*A'*, *A*), day 15 (*B*), day 20 (*C*), and day 30 (*D*). Staining (*small arrows*) was observed in the molecular (*ml*), Purkinje cell (*pc*), and granular cell (*gc*) layers. *pz* indicates the position of the proliferative zone. *Midsize arrows* in *A* mark migrating granule cells, and *large arrows* in *B* indicate a punctate staining present in the molecular layer. Scale bars, 10 μm.

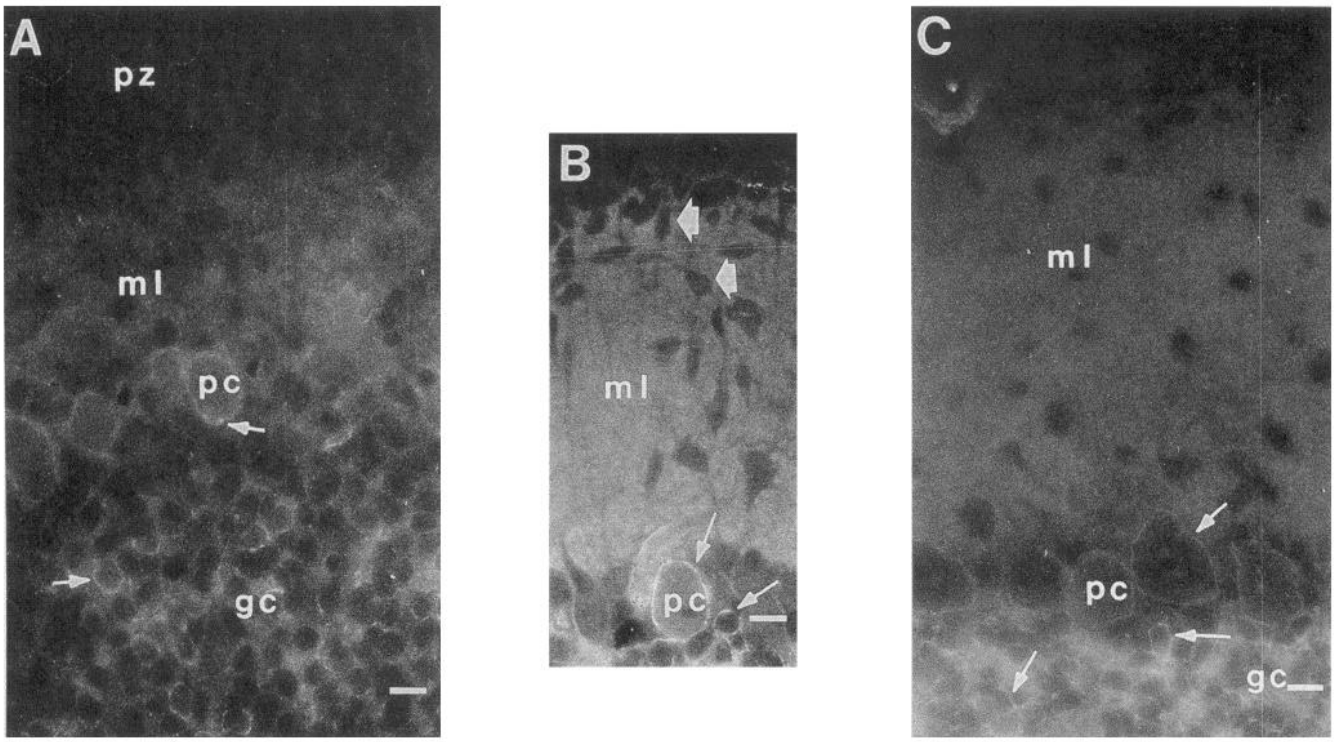

Figure 8. Immunofluorescent localization of β_R-spectrin in the developing rat cerebellum: lateral 4 μm sections of the developing rat cerebellum stained with anti-β_R-spectrin. Micrographs represent postnatal day 10 (*A*), day 15 (*B*), and day 20 (*C*). Staining (*small arrows*) was observed in the molecular (*ml*), Purkinje cell (*pc*), and granular cell (*gc*) layers. *pz* indicates the position of the proliferative zone, and *large arrows* mark migrating granule cells. Scale bars, 10 μm.

cessing; these two regions may interact in altering the function of ankyrin$_R$ in the brain. In parallel, β_R-spectrin is also modified from its erythrocyte form by tissue-specific alternative mRNA processing that takes place in both the brain and skeletal muscle (Winkelmann et al., 1990). The function of these proteins in the rat brain remains to be established.

The temporal expression of ankyrin genes in the rat brain parallels that of its preferred spectrin partner. Ankyrin$_B$ is expressed early in postnatal neuronal development (Kunimoto et al., 1991), similar to β_G-spectrin, for which it has a high-affinity binding site. The molecule is initially present as a 440 kDa protein that is subject to alternative mRNA processing to give rise to a 220 kDa product representing the major brain ankyrin isoform. In this study we show that ankyrin$_R$, like β_R-spectrin, is expressed later in neuronal development. In the developing rat cerebellum, expression of these proteins in granule cells is not observed in mitotic cells of the proliferative zone, or in cells undergoing migration from this zone to the granular cell layer. The expression of ankyrin$_R$ and β_R-spectrin in cells of the granular layer suggests that their expression may be coupled to the terminal stage of neuronal differentiation induced by synaptic contacts from mossy fibers on newly arriving granule cells in this layer.

The postmitotic expression of ankyrin$_R$ and β_R-spectrin suggests that these proteins are involved in membrane remodeling consistent with a role in the formation of specialized membrane domains unique to the maturation stage of this subset of neurons. This could include a role in synaptogenesis or in the interaction between neuronal and glial cells during glial cell encapsulation of Purkinje cells (Altman, 1972).

One potential function for ankyrin$_R$ is suggested by its apparent localization at the light microscopic level to dendritic spines of developing Purkinje cells. These structures represent the postsynaptic terminals for glutamate-mediated neurotransmission via parallel fibers present in the molecular layer of the cerebellar cortex. A role for ankyrin$_R$ and the membrane skeleton in the dendritic spine might lie in the organization of the postsynaptic membrane and the localization of the metabotrophic glutamate receptor to this structure. Siman et al. (1985) have proposed a role for the membrane skeleton in the regulation of glutamate receptor function. The metabotrophic glutamate receptor isoform localized to the Purkinje cell dendritic spine shows a pattern of cellular localization similar to that of ankyrin$_R$, particularly in the basal ganglia, where it is highly enriched in the globus pallidus (Martin et al., 1992).

It remains to be established why this particular subset of neurons in the rat brain should express genes that encode proteins of the erythrocyte membrane skeleton. One possible explanation is that these cells derive from the same group of embryonic precursors and hence share common developmental characteristics. However, this explanation seems unlikely given the widespread distribution of cells expressing ankyrin$_R$ and β_R-spectrin. A second possibility is shared structural or biochemical characteristics among these cells, such as the expression of a particular neurotransmitter receptor. The high level of ankyrin$_R$ and β_R-spectrin expression in the metencephalon, and in particular in the Purkinje cells of the cerebellar cortex, suggests that these cells may share structural or biochemical features with the subset of forebrain neurons that express these proteins. Evidence for shared biochemical features between Purkinje cells and neurons of certain thalamic nuclei has been noted in a mutant strain of mice (O'Gorman and Sidman, 1985). These mice have a

deficiency of glutamate-stimulated inositol phospholipid accumulation and Purkinje cell degeneration (Blackstone et al., 1989). Ankyrin$_R$ deficiency has also been associated with Purkinje cell degeneration in the *nb* mutation, although whether this phenomenon extends to ankyrin$_R$-expressing cells in the forebrain has not been reported.

The late onset and restricted localization of ankyrin$_R$ and β_R-spectrin expression suggest that these molecules could have a role in human cognitive disorders. Parallels may be made with another structural protein, dystrophin, which shows a similar pattern of restricted expression in the brain (Lidov et al., 1990). In patients with muscular dystrophy due to dystrophin deficiency, a preferential loss of these select neuronal populations is observed (Rosman and Kakulas, 1966) that may account for some of the severe cognitive and behavioral disabilities often associated with the disease (Whelan, 1987). In the case of ankyrin$_R$, neurological abnormalities have been observed in a subset of patients with hereditary spherocytosis, where the underlying cause of aberrant red cell morphology was a deficiency of ankyrin$_R$ (Bennett and Lambert, 1991).

References

Altman J (1972) Postnatal development of the cerebellar cortex in the rat. Phases in the maturation of Purkinje cells and of the molecular layer. J Comp Neurol 145:399–464.

Bennett V (1983) Erythrocyte membrane proteins: interaction of ankyrin with spectrin and band 3. Methods Enzymol 96:313–324.

Bennett V (1990) Spectrin-based membrane skeleton: a multipotential adaptor between plasma membrane and cytoplasm. Physiol Rev 70: 1029–1065.

Bennett V (1992) Ankyrins. J Biol Chem 267:8703–8706.

Bennett V, Lambert S (1991) The spectrin skeleton: from red cells to brain. J Clin Invest 87:1483–1489.

Blackstone G, Supattone S, Snyder S (1989) Inositol phospholipid–linked glutamate receptors mediate cerebellar parallel-fiber–Purkinje-cell synaptic transmission. Proc Natl Acad Sci USA 86:4316–4320.

Davis J, Bennett V (1983) Brain-spectrin: isolation of subunits and formation of hybrids with erythrocyte spectrin subunits. J Biol Chem 258:7757–7766.

Davis L, Bennett V (1990) Mapping the binding sites of human erythrocyte ankyrin for the anion exchanger and spectrin. J Biol Chem 265:10589–10596.

Davis L, Otto E, Bennett V (1991) Specific 33-residue repeat(s) of erythrocyte ankyrin associate with the anion exchanger. J Biol Chem 266:11163–11169.

Davis L, Davis J, Bennett V (1992) Ankyrin regulation: an alternatively spliced segment of the regulatory domain functions as an intramolecular modulator. J Biol Chem 267:18966–18972.

Hall T, Bennett V (1987) Regulatory domains of erythrocyte ankyrin. J Biol Chem 262:10537–10545.

Henkel T, Zabel V, VanZee K, Muller J, Fanning E, Bauerle P (1992) Intramolecular masking of the nuclear location signal and dimerization domains in the precursor for the p50 NF-kB subunit. Cell 68: 1121–1133.

Hu R-J, Watanabe M, Bennett V (1992) Characterization of human brain cDNA encoding the general isoform of beta spectrin. J Biol Chem 267:18715–18722.

Kordeli E, Bennett V (1991) Distinct ankyrin isoforms at neuron cell bodies and nodes of ranvier resolved using ankyrin$_R$-deficient mice. J Cell Biol 114:1243–1260.

Kordeli E, Davis J, Trapp B, Bennett V (1990) An isoform of ankyrin is localized at the nodes of Ranvier in myelinated axons of central and peripheral nerves. J Cell Biol 110:1341–1352.

Kunimoto M, Otto E, Bennett V (1991) A new 440-kD isoform is the major ankyrin in neonatal rat brain. J Biol Chem 115:1319–1331.

Lambert S, Prchal J, Lawler J, Ruff P, Speicher D, Cheung M, Kan Y, Palek J (1990) cDNA sequence for human erythrocyte ankyrin. Proc Natl Acad Sci USA 87:1730–1734.

Lidov H, Byers T, Watkins S, Kunkel L (1990) Localization of dystrophin to post-synaptic regions of central nervous system cortical neurons. Nature 348:725–728.

Lux S, John K, Bennett V (1990a) Analysis of cDNA for human rbc ankyrin indicates a repeated structure with homology to tissue-differentiation and cell-cycle control proteins. Nature 344:36–42.

Lux S, Tse W, Menninger J, John K, Harris P, Shaleu O, Chilcote R, Marchesi S, Watkins P, Bennett V, McIntosh S, Collins F, Francke U, Ward P, Forget B (1990b) Hereditary spherocytosis associated with deletion of human erythrocyte ankyrin gene on chromosome 8. Nature 345:736–739.

Martin LJ, Blackstone CD, Huganir RL, Price DL (1992) Cellular localization of a metabotropic glutamate receptor in rat brain. Neuron 9:259–270.

Michaely P, Bennett V (1992) The Ank repeat: a ubiquitous motif involved in macromolecular recognition. Trends Cell Biol 2:127–129.

Nelson J, Lazarides E (1984) The patterns of expression of two ankyrin isoforms demonstrate distinct steps in the assembly of the membrane skeleton in neuronal morphogenesis. Cell 39:309–320.

O'Gorman S, Sidman R (1985) Degeneration of thalamic neurons in "Purkinje cell degeneration" mutant mice. I: Distribution of neuron loss. J Comp Neurol 234:277–297.

Otto E, Kunimoto M, Mclaughlin T, Bennett V (1991) Isolation and characterization of cDNA's encoding human brain ankyrins reveal a family of alternatively spliced genes. J Cell Biol 114:241–253.

Palek J, Lambert S (1990) Genetics of the red cell membrane skeleton. Semin Hematol 27:290–332.

Peters L, Birkenmeier C, Bronson R, White R, Lux S, Otto E, Bennett V, Higgins A, Barker J (1991) Erythroid ankyrin gene expression in normal and ankyrin-deficient mice: the normoblastosis mutation causes a neurological disorder related to erythroid ankyrin deficiency in the brain. J Cell Biol 114:1233–1242.

Repasky E, Symer D, Bankert R (1984) Spectrin immunofluorescence distinguishes a population of naturally capped lymphocytes *in situ*. J Cell Biol 99:350–355.

Riederer B, Zagon I, Goodman S (1986) Brain spectrin (240/235) and brain spectrin (240/235E), two distinct spectrin subtypes with different locations within mammalian neural cells. J Cell Biol 102:2088–2097.

Riederer B, Zagon I, Goodman S (1987) Brain spectrin (240/235) and brain spectrin (240/235E) differential expression during mouse brain development. J Neurosci 7:864–874.

Rosman N, Kakulas B (1966) Mental deficiency associated with muscular dystrophy. A neuropathological study. Brain 89:769–788.

Siman R, Baudry M, Lynch G (1985) Regulation of glutamate receptor binding by the cytoskeletal protein fodrin. Nature 313:225–228.

Siman R, Ahdoot M, Lynch G (1987) Ontogeny, compartmentation, and turnover of spectrin isoforms in rat central neurons. J Neurosci 7:55–64.

Southern E (1975) Detection of specific sequences among DNA fragments separated by electrophoresis. J Mol Biol 98:503–517.

Srinivasan Y, Lewallen M, Angelides K (1992) Mapping the binding site on ankyrin for the voltage-dependent sodium channel from brain. J Biol Chem 267:7483–7489.

Studier F, Rosenberg A, Dunn J, Dubendorf J (1990) Use of T7 RNA polmerase to direct expression of cloned genes. Methods Enzymol 185:62–89.

Tse W, Menninger J, Yang-Feng T, Francke U, Sahr K, Lux S, Ward D, Forget B (1991) Isolation and chromosomal localization of a novel nonerythroid ankyrin gene. Genomics 10:858–866.

Tsuji A, Kawasaki K, Ohnishi S, Merkle H, Kusumi A (1988) Regulation of band 3 mobilities in erythrocyte ghost membranes by protein association and cytoskeletal meshwork. Biochemistry 27:7447–7452.

Whelan T (1987) Neuropsychological performance of children with Duchenne muscular dystrophy and spinal muscle atrophy. Dev Med Child Neurol 29:212–220.

Winkelmann J, Costa F, Lingie B, Forget B (1990) Beta-spectrin in human skeletal muscle. J Biol Chem 265:20449–20454.