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Molecular Cloning and Developmental Analysis of a New Glutamate Receptor Subunit Isoform in Cerebellum

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The glutamate receptor gene GluR-4 is proposed to generate two spliced isoforms (Sommer et al., 1990). Screening a rat cerebellar cDNA library, we have now identified a third type of transcript derived from GluR-4 gene by differential RNA processing. This transcript encodes a protein with a "flop" module between transmembrane regions 3 and 4, but with a C-terminus segment of 36 amino acids different from the previously described GluR-4 flip/flop cDNAs. This subunit was therefore designated as GluR-4c flop. Transcripts synthesized *in vitro* from GluR-4c cDNA form kainate/AMPA-activated channels when expressed in *Xenopus* oocytes. The current–voltage relationship for kainate-evoked responses in oocytes injected with GluR-4c showed strong inward rectification. The different transcripts derived from the GluR-4 gene were studied on Northern blots hybridized with either a cDNA probe or oligonucleotides specific for the GluR-4 flip/flop and C-terminal domains. Three transcripts of 6.2, 4.2, and 3.0 kilobases (kb) derived from the GluR-4 gene were identified on Northern blots containing total RNA prepared from different brain regions, using a cDNA probe or an oligonucleotide corresponding to the N-terminal region common to all transcripts. These transcripts were much more abundant in the cerebellum than in other brain areas, and their levels increased during cerebellar development. The maximal increase was observed between postnatal days 1 and 20, an age corresponding to the division and maturation of granule neurons. The flip/flop and the C-terminal oligonucleotides hybridized to the two higher molecular weight transcripts but did not hybridize to the small RNA. Interestingly, using cerebellar cells that were cultured for up to 12 d, we observed that the three transcripts are present in granule neurons, but that astrocytes only express the 6.2 and the 4.2 kb transcripts. The 3.0 kb transcript accumulates in cerebellar granule cells during development *in vitro*. Furthermore, *in situ* hybridization histochemistry revealed that the GluR-4c transcripts are preferentially expressed in cerebellar granule cells and Bergmann glial cells, whereas the expression of GluR-4 flip mRNAs is restricted to Bergmann glial cells. Interestingly, we also show that granule cells already express GluR-4c in the premigratory zone of the external granular layer, indicating that intrinsic or highly localized cues induce GluR-4c expression before these cells reach their final position.

The recent molecular cloning of cDNAs encoding for glutamate-gated channels (Hollmann et al., 1989; Boulter et al., 1990; Keinänen et al., 1990; Nakanishi et al., 1990; Sommer et al., 1990) has shed a new light on the original classification of excitatory amino acid receptors into three subtypes based on the "selective" agonist activation by NMDA, kainate, and quisqualate (Watkins and Evans, 1981). Four glutamate receptor subunits cloned so far (GluR-1 to GluR-4) form functional membrane channels when expressed in oocytes or in transfected cells, and are activated by the non-NMDA receptor agonists kainate, AMPA, and quisqualate (Hollmann et al., 1989; Boulter et al., 1990; Keinänen et al., 1990; Nakanishi et al., 1990; Sommer et al., 1990). In addition, a fifth subunit (GluR-5) cloned by Bettler et al. (1990) forms functional channels, but these are only weakly responsive to L-glutamate. The functional properties of glutamate-gated channels appear to depend on the type of subunit combinations expressed (Boulter et al., 1990; Keinänen et al., 1990; Nakanishi et al., 1990).

Importantly, Sommer et al. (1990) have cloned two alternative spliced isoforms for GluR-1, -2, -3, and -4 (designated "flip" and "flop") that differ from each other in a small segment of 38 amino acids that lie between the third and fourth putative transmembrane domains (M3, M4). These amino acid sequences confer different kinetic properties to glutamate-gated channels (Sommer et al., 1990), indicating that the functional properties of the native receptor channel in a cell are defined by the type of subunit combination and splice variant. The finding that the flip and flop isoforms are differentially distributed (Sommer et al., 1990) and regulated during development (Monyer et al., 1991) suggests that they may have unique functions.

GluR-1, -2, -3, and -4 have been shown to be highly expressed in the cerebellum (Hollmann et al., 1989; Sommer et al., 1990), which serves as an excellent model to determine the molecular composition of glutamate receptors in specific cell types and to study their expression during development. Among the different brain areas, the cerebellar cortex is one of the best understood in terms of cell types, structure, and synaptic circuits (Palay and Chan-Palay, 1974; Ito, 1984). In the cerebellar cortex, glu-

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tamate mediates neurotransmission between granule cells and Purkinje cells, and between mossy fiber terminals and granule cell dendrites (Gallo et al., 1982; Somogyi et al., 1986; Dupont et al., 1987; Garthwaite and Brodbelt, 1989; D'Angelo et al., 1990). Since some of the cerebellar cell populations develop postnatally, it is possible to obtain primary cultures from rat cerebellum enriched in different neural cell types (Aloisi et al., 1985; Kingsbury et al., 1985; Gallo and Bertolotto, 1990). This has allowed a detailed study of the pharmacological and functional properties of the native glutamate receptor channels in cerebellar neurons (Gallo et al., 1987, 1990; Cull-Candy et al., 1988; Howe et al., 1991), as well as in glial cells (Gallo et al., 1986, 1989a; Usowicz et al., 1989; Wyllie et al., 1991). This analysis has shown that both granule neurons and astrocytes in culture express kainate/AMPA receptors and channels with similar properties (Gallo et al., 1987, 1989a,b, 1990; Cull-Candy et al., 1988; Usowicz et al., 1989; Howe et al., 1991; Wyllie et al., 1991). However, it still remains to be determined whether the similarities between neuronal and astrocytic channels (Usowicz et al., 1989) correspond, at the molecular level, to similar subunit combinations.

We have used the GluR-1 nucleotide sequence (Hollmann et al., 1989) to identify cDNAs coding for additional GluR subunits, with the aim of studying their developmental regulation in different cerebellar cell types *in vivo* and in culture. In the present study, we report the isolation of a cDNA clone, GluR-4c, from a rat cerebellar library that is more than 90% homologous to GluR-4 (Bettler et al., 1990; Keinänen et al., 1990) but differs from its sequence in a contiguous region of 36 amino acids located in the C-terminus of the protein. We provide evidence that GluR-4c is functional when expressed in *Xenopus* oocytes and we present the first detailed electrophysiological characterization of transcripts derived from the GluR-4 gene. In addition, we demonstrate that GluR-4 transcripts are present both in cultured cerebellar neurons and astrocytes, and that one of these transcripts is specifically expressed and developmentally regulated in granule cells. The facts that multiple clones coding for the different C-terminus domain were obtained, that oligonucleotides specific to this region hybridized to stable transcripts, and that the 36 amino acid terminal sequence is homologous to two other glutamate receptor subunits demonstrate that GluR-4 transcripts are differentially processed, not only in the flip/flop region, but also at their C-terminal domain.

Materials and Methods

Isolation of GluR-4c cDNA. Total RNA was isolated from cerebella of adult Sprague–Dawley rats as previously described (Chirgwin et al., 1979). PolyA$^+$ RNA was purified on oligo(dT)-cellulose column (Pharmacia), and a directional cDNA library was constructed in the bacteriophage expression vector λZAPII, using the Stratagene cDNA synthesis kit (Stratagene, La Jolla, CA). A total of 2×10^5 plaques were screened from the primary library containing 1.2×10^6 independent clones. Two oligonucleotides were synthesized with sequences derived from the GluR-1 cDNA sequence published by Hollmann et al. (1989). The sequence corresponded to the regions coding for the putative transmembrane domain M2 (nucleotides 1561–1608) and M4 (nucleotides 2362–2409). The probes were end-labeled with α-^{32}P-ATP and T4 polynucleotide kinase (10^7 cpm/pmol). Duplicate filters containing recombinant plaques were hybridized with either the M2 or the M4 probes at low stringency [6× SSC (1× SSC: 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7.0), 0.1% SDS, 5× Denhart's, 10 mM EDTA, 100 μg/ml salmon sperm DNA, 60°C]. The filters were sequentially washed at increasing stringencies (6× SSC, 0.1% SDS, 55°C; 3× SSC, 0.1% SDS, 65°C; 0.3× SSC, 0.1% SDS, 65°) and exposed to film, so that cDNAs with increasing homology to GluR-1 could be identified. A third oligonucleotide, made to the 3′ untranslated region (nucleotides 2695–2715), was used to screen out GluR-1–positive clones. More than 70 positive clones were isolated, and several of them were further characterized (see Results). The primary cerebellar cDNA library was rescreened for additional GluR-4c clones by using an oligonucleotide probe specific to its C-terminus (oligonucleotide D). Filters containing recombinant plaques (1.6×10^5 total) were hybridized at low stringency (6× SSC, 0.1% SDS, 5× Denhart's, 10 mM EDTA, 100 μg/ml salmon sperm DNA, 60°C). The filters were sequentially washed at increasing stringencies (0.1× SSC, 0.1% SDS, 55°C; 0.1× SSC, 0.1% SDS, 60°C) and exposed to film. Two positive clones were identified. The same filters were subsequently stripped and then rehybridized with an oligonucleotide probe for the C-terminus domain found in the previously published GluR-4 cDNAs (5′-TGGTAGGTCCGATGCAATGACAGCCAATCCCGAACTTTGTCTAATTGCAG-3′). The filters were washed at high stringency (0.1× SSC, 0.1% SDS, 60°C), and a total of 14 clones were hybridized.

Nucleotide and deduced amino acid sequence analysis. Partial nucleotide sequence was obtained from the GluR-4c cDNA, which was excised *in vivo* into the pBluescript II SK vector, by creating serial deletions with the DNA Exonuclease III Exometh Kit (Stratagene). This information was then used to generate internal oligonucleotide primers to sequence the double-stranded cDNA on both strands by the Sanger dideoxy-termination technique (Sanger et al., 1977). Two additional GluR-4c cDNAs were sequenced in the C-terminus region with oligonucleotides used to sequence the initial GluR-4c clone. Nucleotide sequences and deduced amino acid sequences were assembled and analyzed by using the DNASTAR package (DNASTAR, Inc., Madison, WI).

In vitro *synthesis of GluR-4c transcripts and expression in oocytes.* pGluR-4c DNA was linearized with XhoI endonuclease, digested with proteinase K (10 μg/ml), phenol/chloroform extracted twice, and finally extracted in chloroform. Transcription reactions (25 μl) contained (mCAP mRNA capping kit, Stratagene) 40 mM Tris-HCl (pH 7.5); 50 mM NaCl; 8 mM $MgCl_2$; 2 mM spermidine; 0.4 mM (each) rUTP, rCTP, rATP, and rGTP; 30 mM dithiothreitol (DTT), 1 mM 5′7meGppp5′G; 25 U of T3 DNA polymerase (Stratagene); and 2 μg of linearized plasmid DNA. Reactions were incubated at 37°C for 30 min, and the DNA template was subsequently digested with 10 U of RNase-free DNase I (Stratagene). RNA transcripts were precipitated in ammonium acetate and ethanol. The quantity of the synthesized RNA was determined spectrophotometrically.

Oocytes were dissected from adult anesthetized (0.1% aminobenzoic acid ethylester) *Xenopus laevis* and defolliculated with collagenase type 1A (1.5 mg/ml; Boehringer-Mannheim) in OR2 medium (no Ca^{2+}) and kept in modified Barth's solution [in mM: 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 15 HEPES (pH 7.6), 0.3 $CaNO_3$, 0.41 $CaCl_2$, 0.82 $MgSO_4$, 10 μg/ml penicillin, and 10 μg/ml streptomycin] at 18°C before and after the injection. The oocytes were injected with 10–20 ng of *in vitro*–transcribed RNAs (10–50 nl vol); media were exchanged daily. Electrophysiological experiments were performed typically 4–6 d after injection using a continuously perfused chamber. Membrane currents elicited by the application of excitatory amino acids dissolved in Barth's solution were recorded using two-electrode voltage clamp (Axoclamp 2A), with electrodes of 0.5–5 MΩ resistance filled with 1 M KCl or 1 M CsCl. Current responses were filtered at 20 Hz and recorded on a Gould chart recorder. No kainate- or quisqualate-like responses were observed in oocytes injected with saline solution.

Northern blot analysis of GluR-4c transcripts in the developing cerebellum and in cultured cells. Total RNA was isolated from Sprague–Dawley rat cerebella at different developmental stages, from embryonic age 17 (E17) to 6-week-old rats, as well as cultured cells, using the guanidine thiocyanate procedure (Chirgwin et al., 1979). PolyA$^+$ and polyA$^-$ RNAs were purified on an oligo(dT)-cellulose column. RNA samples (15 μg/lane for total RNA; 10 μg/lane of polyA$^+$) were electrophoresed through a 1.5% agarose-formaldehyde denaturing gel and electrotransferred onto Nytran membranes, which were hybridized with either a cDNA fragment or specific oligonucleotide probes. An internal PstI fragment [2.6 kilobases (kb)], labeled by random priming with α-^{32}P-dCTP, was used as a probe (10^8 cpm/μg DNA). When using the cDNA probe, blots were hybridized in 50% formamide at 42°C and washed at high stringency with 0.1× SSC at 65°C. The location [in base pairs (bp)] and sequence of the oligonucleotides used are listed below:

A: 5′-AGCCCTTGAATTCGAACCTGCTT-CAGTGTCCTCTCCATGTCAATTCCC-3′ (1023);
B: 5′-GACGCCTGCCTCACTGAGTTT-CAAAACGGCAAGGTTTACAGGAGTTCT-3′ (2294);

C: 5′-GGCACTCGTCTTGTCCTTGGAGT-CACCTCCCCCGCTGCCACATTCTCC-3′ 2377);
D: 5′-AAGTCTGTGCACTCTTTGCCACCTT-CATTCTCTTCGCCTCTGCCCTGG-3′ (2519);
E: 5′-CTAATTGCAGTTCCTGTGTGTACGGCCTTGG-GGCAGTCAGGGGTCAGC-3′ (2785).

Oligonucleotides, purified on denaturing polyacrylamide gels and end-labeled with terminal transferase to a specific activity of 4×10^6 cpm/pmol by addition of approximately 20 α-^{32}P-dATP molecules at the 3′ end, were hybridized to blots at 55°C in a solution containing 5× SSC, 8× Denhart's, 1% SDS, 8 mM EDTA, 0.08% Na^+-pyrophosphate, and 0.08 mg/ml salmon sperm DNA (4×10^6 cpm/ml) and washed at high stringency (0.1× SSC, 60°C).

Primary cultures. Cerebellar primary cultures were prepared from 8-d postnatal (P8) Sprague–Dawley rats as previously described (Levi et al., 1984). Dissociated cells were plated in 100-mm-diameter plastic dishes (Nunc) precoated with 5 μg/ml poly-L-lysine at a density of 2.5 or 1.2×10^5 cells/cm² (neuronal and astrocytic cultures, respectively). Cells were grown in Basal Eagle's Medium (neuronal cultures) or in D-valine–containing Minimal Essential Medium (MEM) (astrocyte cultures) (GIBCO-BRL, Bethesda, MD), containing 10% fetal calf serum, 2 mM glutamine, and 100 μg/ml gentamicin. The medium for neuronal cultures also contained 25 mM KCl (final concentration). After 16–20 hr, 10 μM cytosine arabinoside was added to the neuronal cultures to prevent the replication of non-neuronal cells. The culture medium was changed to the astrocyte cultures at 3 d *in vitro* and every other day afterward. Cerebellar neuronal cultures comprised more than 95% granule cells (Levi et al., 1984; Kingsbury et al., 1985), whereas astrocyte cultures comprised more than 95% glial fibrillary acid protein (GFAP)–positive cells (type 1 and type 2 astrocytes; Gallo and Bertolotto, 1990) and a small proportion of galactocerebroside-positive oligodendrocytes (2–3%).

In situ *hybridization histochemistry.* Protocols using 12 and 20 μm fresh-frozen rat cerebellar sections cryosectioned parasagittally were followed as described in Hayes and Loh (1990). Briefly, rats at postnatal days 1, 8, 20, and 40 (Taconic Farms, Germantown, MD) were killed using carbon dioxide and decapitated, and cerebella were rapidly dissected and freeze-embedded in Tissue-Tek media (Miles) using dry ice. Sections on gelatin-coated slides were fixed in paraformaldehyde, acetylated in acidified triethanolamine, and delipidated in an ethanol series and chloroform. Oligonucleotides that were 50–60% in GC content were end-tailed with ^{35}S-dATP (New England Nuclear) by incubation with terminal transferase (Boehringer-Mannheim) using standard methods. One hundred microliters of a complex buffer (50% deionized formamide, 4× SSC, 1× Denhart's, 0.25 mg/ml yeast *t*RNA, 0.5 mg/ml sheared single-stranded salmon sperm DNA, 10% dextran sulfate, 50 mM DTT) comprising 1.5×10^6 cpm were pipetted onto six to eight sections per slide, covered with parafilm strips, and incubated in humidified dishes at 37°C for 18–20 hr. Slides were washed at high stringency in 2× SSC/50% formamide at 40°C, further washed, dried, exposed to Hyperfilm (Amersham) for 10–14 d, and then dipped in Kodak NTB-3 emulsion and stored in dark for 4–6 weeks at 4°C. They were then processed in Kodak solutions of Dektol and regular fix at 15°C, stained with 0.2% methyl green, dehydrated, and coverslipped in Permount (Fisher). As control for specificity, the antisense oligonucleotide probes (A, B, C, and D) were shown to hybridize the same bands recognized by the GluR-4c cDNA on Northern blots (see Fig. 3) and showed similar reproducible labelling patterns on tissue sections. Morphological structures in developing cerebella were identified using Altman (1972a,b) and Palay and Chan-Palay (1974).

Results

Isolation and characterization of a cDNA originating from a different GluR-4 gene transcript

A cDNA library of rat cerebellum was screened at increasing stringencies for GluR cDNAs using two oligonucleotide probes coding for the putative transmembrane domains M2 and M4 proposed by Hollmann et al. (1989) for the GluR-1 subunit; more than 70 clones were obtained that hybridized with both or either probe. These clones were subdivided into groups that were either M2 positive (34 clones), M4 positive (15 clones), or M2+M4 positive (30 clones) at the highest stringencies tested (see Materials and Methods). We initially analyzed the three largest cDNAs from the group that were M2+M4 positive but that did not hybridize with a probe to the 3′ untranslated region of GluR-1. Two of these clones differed in a segment of 115 bp residing between the two putative spanning helices M3 and M4, which are generated by alternative splicing and designated by others as the "flip" and "flop" isoforms (Sommer et al., 1990).

We determined by DNA sequencing that the third isolated cDNA is different from either of these isoforms and originates from a different transcript derived from the GluR-4 gene; it has been designated the name GluR-4c flop. For simplicity, here we adopted the nomenclature GluR-1, -2, -3, and -4 (Boulter et al., 1990; Nakanishi et al., 1990), which correspond to GluR-A, -B, -C, and -D, respectively (Keinänen et al., 1990). For the cDNA that we have isolated, the number 4 indicates the glutamate receptor gene type, and the small letter "c," the difference in the C-terminal domain.

The nucleotide sequence of the GluR-4c flop cDNA has an open reading frame that extends 883 amino acids (Fig. 1); the first ATG in the open reading frame was chosen as the initiator methionine because the surrounding sequence (GAAGATG) conforms to a consensus vertebrate translation initiation site (Cavener, 1987). The N-terminal 21 amino acids from the deduced protein sequence have the features characteristic of a signal peptide (Steiner et al., 1980; Fig. 1). While the protein sequence of GluR-4c is virtually identical to the recently published sequence GluR-4 flop (Bettler et al., 1990; Keinänen et al., 1990), there is a great divergence in the last 36 amino acids (849–884) at the C-terminus (Figs. 1, 2*B*). Interestingly, as shown in Figure 2*B*, 25 and 23 of the terminal 36 amino acids in GluR-4c show homology but are not identical to the corresponding C-terminus amino acids present in GluR-2 and -3, respectively (Bettler et al., 1990; Boulter et al., 1990; Keinänen et al., 1990; Nakanishi et al., 1990). Furthermore, in the GluR-4c DNA sequence there are two G-residues located downstream of the termination codon, followed by an open reading frame that codes for identical amino acids present in the C-terminus end (828–881) of the GluR-4 cDNA described previously (Bettler et al., 1990; Keinänen et al., 1990) (Figs. 1, 2*A*). In order to confirm that the GluR-4c cDNA was not the result of an artifact, we rescreened the primary library with oligonucleotide probes specific for the 36 amino acid C-terminus and hybridized Northern blots containing cerebellar RNA with the same probes (see below). Two additional cDNAs of different sizes that code for sequences that are identical at the C-terminus to the original GluR-4c cDNA were obtained. More recently, we have found that one of these cDNAs contains a flip module (data not shown). These results demonstrate that there are two sites at which GluR-4 transcripts can be differentially processed: at the flip/flop and the C-terminal domains.

GluR-4c transcripts form kainate/AMPA channels in oocytes

We examined the functional properties of GluR-4c by voltage clamp analysis of *Xenopus* oocytes injected with GluR-4c transcripts synthesized *in vitro* (Sumikawa et al., 1984). Kainate (100 μM) evoked inward currents in the injected oocytes when the membrane potential was clamped at −60 mV (Fig. 3*A*). Figure 3*A* shows the dose–response curve for the activation of GluR-4c with kainate. The effect of kainate saturated at a concentration of 1 mM and the EC_{50} value obtained using the logistic equation was 44.3 ± 9.3 μM (means ± SEM; $n = 10$). The Hill coefficient from the same curve was 1.3 ± 0.14 (±SD; $n = 10$).

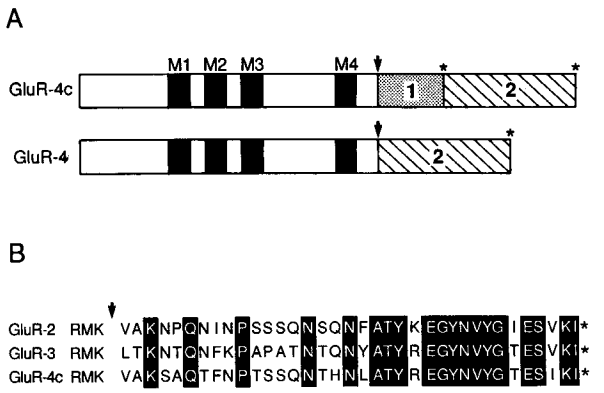

Figure 2. Comparison of different GluR subunit C-termini. *A,* Diagram of the predicted amino acid sequence of GluR-4c and GluR-4. *Boxes 1* and *2* in the C-terminal end denote the 36 amino acids (849–884; *box 1*) that differ from the sequence obtained from GluR-4 cDNAs previously published (Bettler et al., 1990; Keinänen et al., 1990) and the C-terminus of GluR-4 (*box 2*) previously published (Bettler et al., 1990; Keinänen et al., 1990). The four proposed transmembrane domains (M1–M4) are represented in *black. Asterisks* indicate stop codons. A minor difference between GluR-4c and GluR-4 (Keinänen et al., 1990) was in amino acid 713: isoleucine in GluR-4c cDNA instead of threonine (see also Bettler et al., 1990). *B,* Alignment of partial amino acid sequence of GluR-2, GluR-3, and GluR-4c in the C-terminus region of the mature protein. *Asterisks* indicate stop codons, and the *arrow* denotes the beginning of the 36 amino acid segment that differs between GluR-4c and GluR-2 and -3 (see also *A*). Identical amino acids between GluR-4c and GluR-2 and -3 are shown in a *black background.*

We examined the current–voltage (I/V) relationship for kainate-evoked responses in oocytes injected with GluR-4c. Figure 3*B* shows strong inward rectification, similar to that described for GluR-1 and -3 (Boulter et al., 1990): at membrane potentials between −100 and −60 mV the slope is nearly linear, but with further depolarization the slope decreases. At membrane potentials positive to 0 mV no outward current was detected. Oocytes injected with GluR-4c RNA also responded to glutamate, quisqualate, and AMPA, but showed no responses to NMDA (100 μM) in the presence of 3 μM glycine (not shown). The large inward currents generated by the application of kainate (100 μM) were strongly attenuated by coapplication of the non-NMDA receptor antagonist CNQX (0.1–10 μM) or by quisqualate (0.3–30 μM) in a concentration-dependent way (C. A. Winters, L. Vyklicky, and V. Gallo, unpublished observations). Uninjected oocytes did not show responses to glutamate or its analogs.

Complexity of transcripts coded by the GluR-4 gene revealed by Northern blot analysis

Northern blot analysis of total RNA prepared from different brain regions (cerebellum, hippocampus, cerebral cortex, thalamus, and spinal cord) was performed by using a fragment of the GluR-4c cDNA or with oligonucleotide probes corresponding to different regions of the isolated cDNA. The hybridization pattern at high stringency revealed three bands of 6.2, 4.2, and 3.0 kb, respectively (Fig. 4). These transcripts were significantly more abundant in the cerebellum than in the hippocampus, cortex, thalamus, and spinal cord. In the thalamus, hybridization was primarily to the 3.0 kb band. The signals obtained on the blots were specific for RNA isolated from CNS since no hybridization was observed in any of the peripheral tissues examined: liver, muscle, kidney, and spleen (Fig. 4).

The cDNA or oligonucleotide probes used in the experiment described above did not distinguish between the GluR-4 flip/flop and GluR-4c transcripts. Therefore, we designed oligonucleotide probes that selectively hybridized to transcripts coding for either GluR-4c, GluR-4 flip, or GluR-4 flop. Their hybrid-

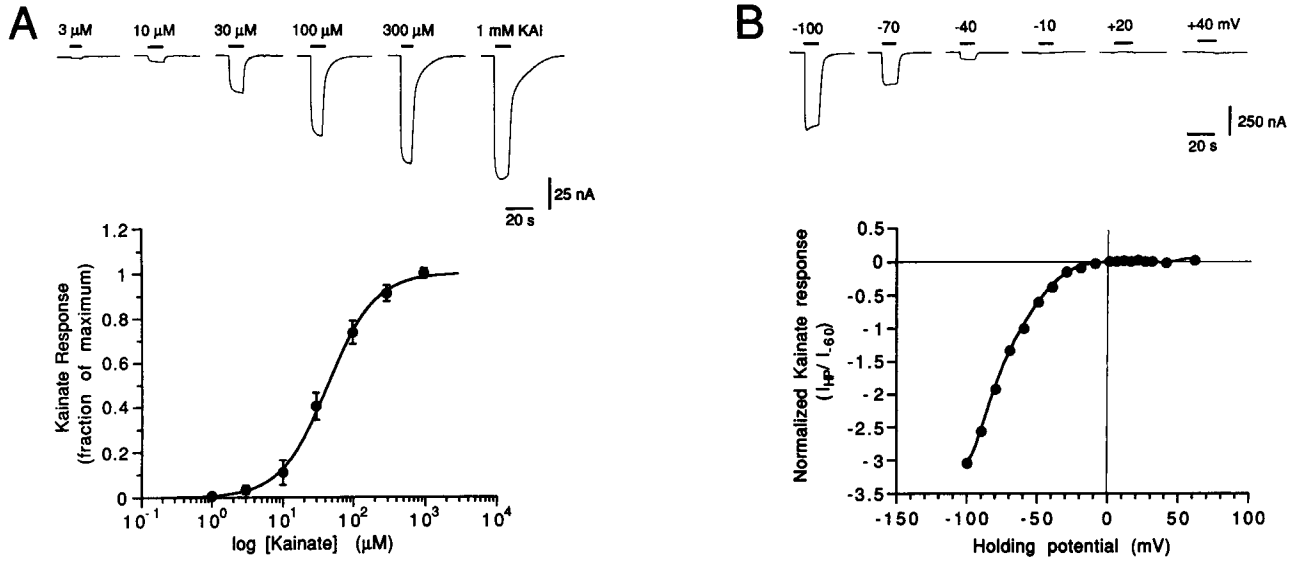

Figure 3. GluR-4c transcripts code for functional kainate/AMPA receptors in *Xenopus* oocytes. *A,* Dose–response curve of GluR-4c for kainate. The *upper part* shows typical inward current responses generated by six different concentrations of kainate in a single oocyte. In the *bottom part,* the responses were normalized with respect to the maximum current (I_{max}) derived from analysis using the logistic equation $I = I_{max} \times 1/1 + (EC_{50}/[dose])^n$. Each point represents the mean ± SD of kainate-evoked currents averaged from 10 oocytes voltage clamped at −60 mV. *B,* Current–voltage plot of kainate current responses in oocytes after injection of GluR-4c. Responses to the application of 100 μM kainate were recorded at different holding potentials in injected oocytes. Each response was normalized to the kainate-evoked current at −60 mV. The curve represents the summary data obtained from 11 oocytes.

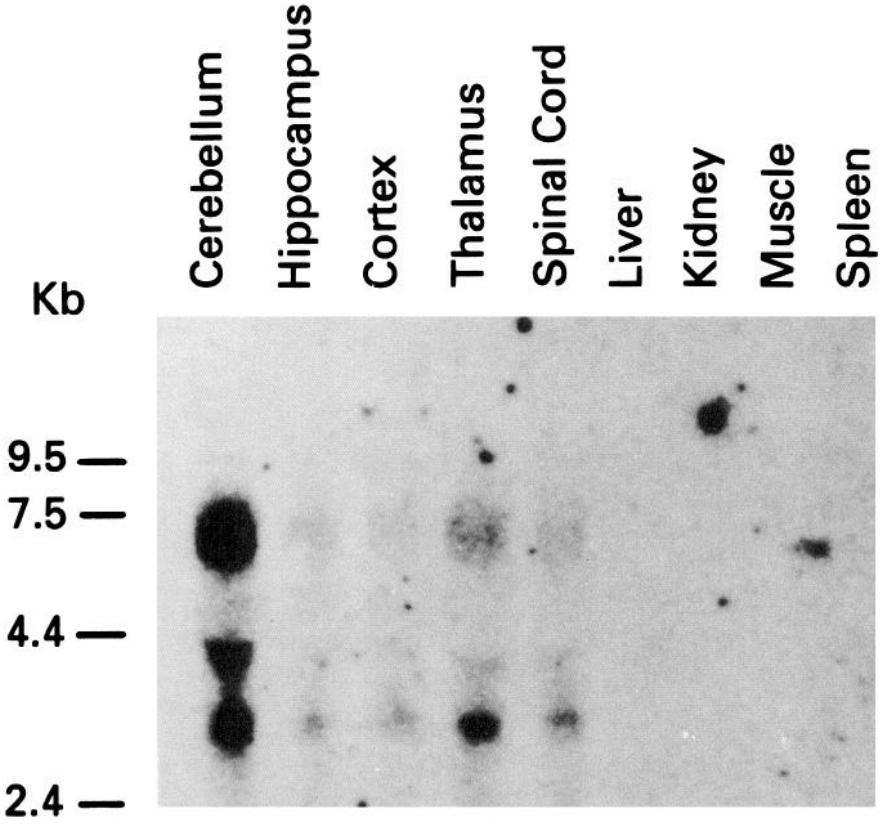

Figure 4. GluR-4 transcripts are more abundant in the cerebellum than in other brain areas. Northern blot analysis of neural RNAs reveals a complex pattern of GluR-4 transcripts that are predominantly expressed in cerebellum. Total RNAs (15 μg/lane) from neural and non-neural tissues, hybridized with a 2.6 kb PstI fragment obtained from the GluR-4c cDNA, show that GluR-4 transcript levels are higher in the cerebellum than in hippocampus, cortex, thalamus, and spinal cord, and are not expressed in non-neural tissues. The same results were obtained with a mixture of ^{32}P-end-labeled oligonucleotide probes A, C, and E. The *numbers* on the *left* represent the sizes in kilobases derived from an RNA standard.

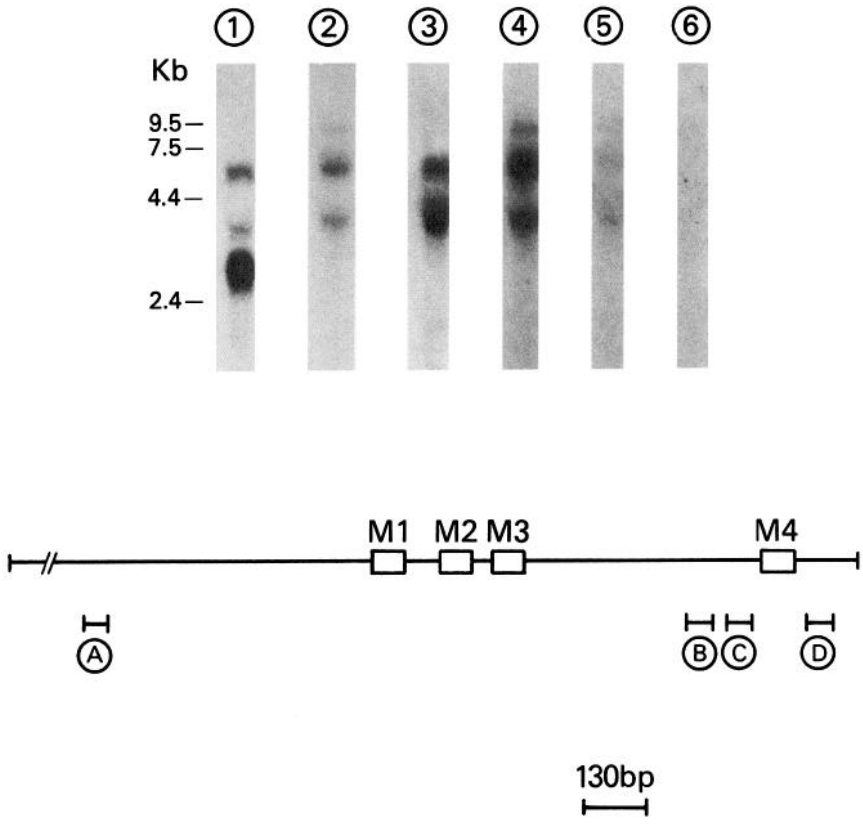

Figure 5. Analysis of different GluR-4 transcripts expressed in the cerebellum with specific oligonucleotide probes. Oligonucleotides specific for either GluR-4c, GluR-4 flip, GluR flop, or a pan-oligonucleotide were hybridized to Northern blots containing cerebellar polyA$^+$, polyA$^-$, and total RNAs. Total RNA (15 μg/lane), polyA$^+$ (10 μg/lane), and polyA$^-$ (20 μg/lane) RNAs, hybridized with ^{32}P-end-labeled oligonucleotide probes, show that only the pan-oligonucleotide probe A labels the low molecular weight transcripts (3.0 kb), whereas all the other probes only label the 6.2 and the 4.2 kb bands. *Lane 1,* polyA$^+$ RNA, pan-oligonucleotide (oligonucleotide A); *lane 2,* polyA$^+$ RNA, flip-specific oligonucleotide (oligonucleotide B); *lane 3,* polyA$^+$ RNA, flop-specific oligonucleotide (oligonucleotide C); *lane 4,* polyA$^+$ RNA, GluR-4c–specific oligonucleotide (oligonucleotide D); *lane 5,* total RNA, oligonucleotide D; *lane 6,* polyA$^-$ RNA, oligonucleotide D. A map of the oligonucleotide probes used is shown in the *bottom. M1–M4,* transmembrane regions. The *numbers* on the *left* represent the sizes in kilobases derived from an RNA standard.

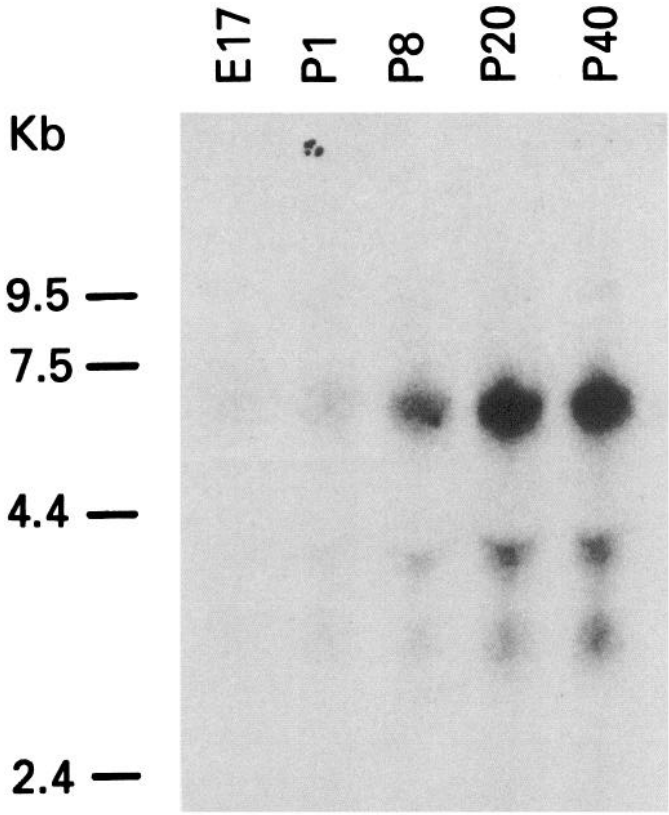

Figure 6. Differential expression of GluR-4 transcripts during cerebellar development. Total rat cerebellar RNA (15 μg/lane) isolated at different developmental stages, probed either with a mixture of the oligonucleotide probes A (pan-oligonucleotide), B (flop-specific oligonucleotide), and E (3′-end oligonucleotide) or with a PstI fragment cDNA GluR-4c probe, shows that GluR-4 transcript levels are very low at E17 and P1. They begin to increase significantly by P8 and P20 and reach steady levels by P40. RNA molecular weight standards are indicated on the *left.*

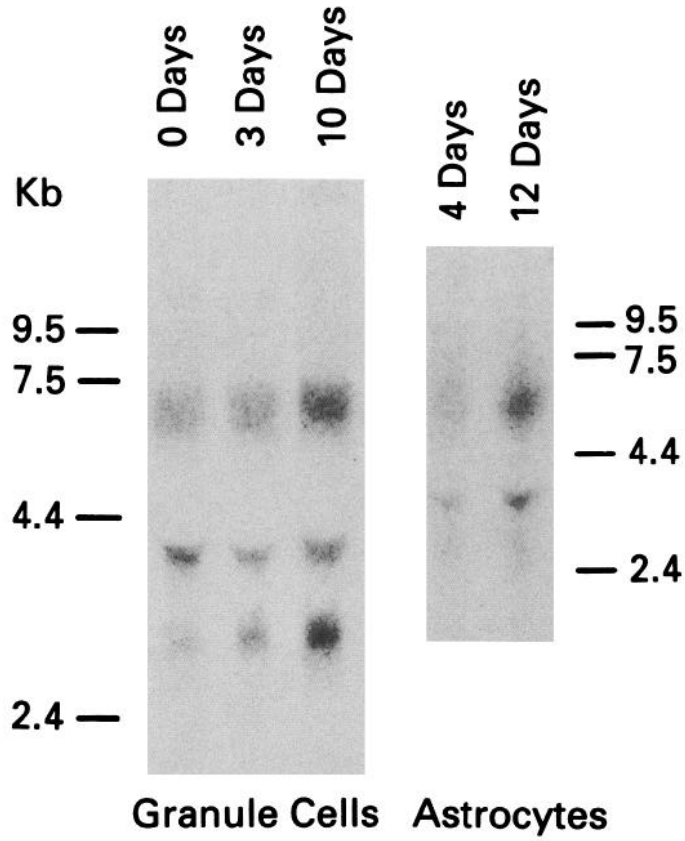

Figure 7. Differential expression of GluR-4 transcripts in different cerebellar neural cell populations in culture. Blots containing 15 μg of total RNA, isolated from cerebellar granule cell and astrocyte cultures at different times *in vitro* were hybridized with a mixture of ^{32}P-ATP–labeled oligonucleotide probes A (pan-nucleotide), B (flop-specific oligonucleotide), and E (3′-end oligonucleotide). The 3.0 kb band is absent in cultured astrocytes but is present in cerebellar granule cells and increases during development in culture. RNA molecular weight standards are indicated on the *left. 0 days,* cells 2 hr after plating.

ization patterns on adult rat polyA$^+$ cerebellar RNA are shown in Figure 5. A pan-oligonucleotide probe (oligonucleotide A, corresponding to a sequence present in GluR-4c and GluR-4 flip/flop) hybridized to three bands at 6.2, 4.2, and 3.0 kb (Fig. 5, lane 1). A quantitative analysis of this hybridization pattern indicated that the intensity of the 3.0 kb band was approximately threefold higher than the 6.2 kb band. An identical hybridization pattern was obtained with a second pan-oligonucleotide located 68 bp upstream of oligonucleotide A (data not shown). The oligonucleotide probes specific for either the flip (oligonucleotide B) or the flop (oligonucleotide C) module and for the GluR-4c 3′-terminus (oligonucleotide D) hybridized to the 6.2 and 4.2 kb bands, but not to the 3.0 kb band (Fig. 5). The specificity of each individual oligonucleotide probe was tested on *in vitro*–synthesized RNA transcripts coding for either the flip, flop or GluR-4c terminal domain. Each of the oligonucleotides B, C, and D hybridized to the specific transcripts, whereas the pan-oligonucleotide labeled all RNAs (data not shown). Finally, the hybridizing bands are specific, because the mRNAs that hybridize with the oligonucleotide probes correspond to those hybridizing with a cDNA probe at high stringency (Fig. 4). Total cerebellar RNA gave the same type of signals at a lower intensity and polyA$^-$ RNA showed no traces of hybridization (Fig. 5).

Developmental expression of GluR-4 gene transcripts in vivo *and in primary cultures*

The relative GluR-4 mRNA levels were measured during cerebellar development from E17 to P40 using Northern blot analysis. Hybridization of blots containing total cerebellar RNA with a cDNA probe or with a mixture of three oligonucleotide probes (A, C, and E) showed that the levels of the GluR-4 transcripts at 6.2, 4.2, and 3.0 kb increase during cerebellar development: the largest increase occurs between P1 and P8 (Fig. 6). In view of the developmental changes observed *in vivo,* we analyzed the expression of GluR-4 transcripts in cerebellar granule cell and astrocyte cultures. The same bands that were observed in the analysis of total cerebellar RNA were also seen in RNA derived from cultured cerebellar granule cells (Fig. 7). The transcripts were present in granule cells cultured for only 2 hr and their expression was maintained up to 10 d *in vitro* (Fig. 7). We have consistently observed that, although the qualitative pattern of the three transcripts does not change between 0 and 10 d *in vitro,* the relative intensity of two of the three bands changes during the culturing period. The levels of the lower molecular weight transcript increase by approximately fivefold throughout the 10 d in culture, while the 6.2 kb transcripts accumulate between days 3 and 10. A similar analysis

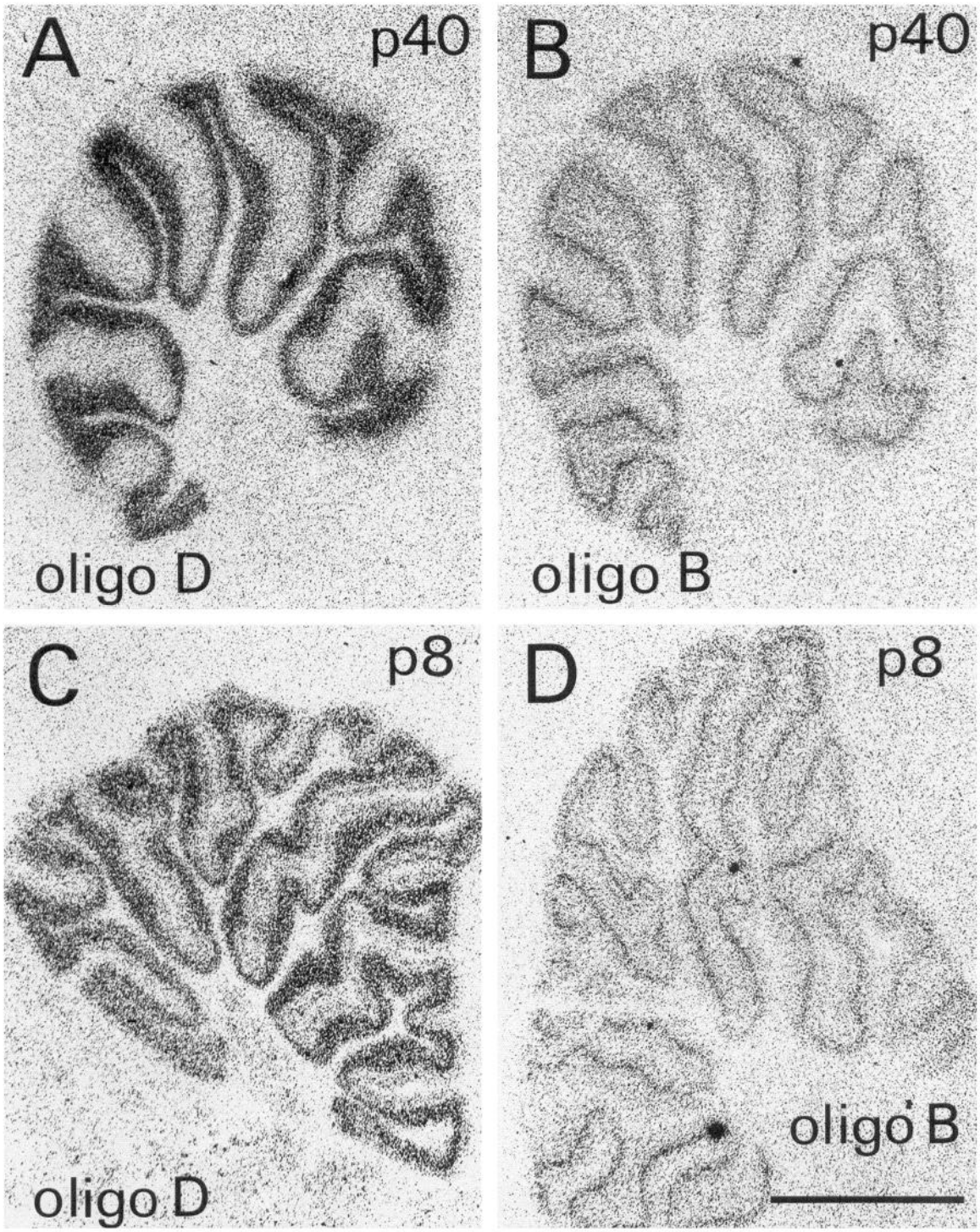

Figure 8. Differential distribution of GluR-4 mRNAs in cerebellar cortical layers during development. Film autoradiography of 20 μm sections from P8 and P40 rat cerebellum, hybridized with an oligonucleotide probe specific for GluR-4c (*A, C*), shows a pattern that differs from that obtained with a GluR flip probe (*B, D*). The distribution of GluR-4c, with wide bands of labeling (*A, C*) both in the Purkinje and internal granule cell layers, is strikingly different from GluR-4 flip, whose pattern consists of narrow bands of labeling that delineate the Purkinje cell layer. Note also for GluR-4c, but not GluR-4 flip, the intense labeling in the internal granular layer at P8 and also parallel bands more lightly labeled corresponding to the innermost regions of the premigratory external germinal layer (see Fig. 9). Scale bar, 1.5 mm (15×).

of cerebellar astrocyte RNA (Fig. 7) showed that the 3.0 kb band was virtually undetectable, whereas GluR-4 probes hybridize with transcripts at 6.2 and 4.2 kb. The relative levels of both transcripts in astrocytes increase during longer times in culture (Fig. 7).

In situ *hybridization studies in developing cerebellum of GluR-4 transcripts*

Localization of GluR-4 transcripts in the developing and adult rat cerebellum by *in situ* hybridization histochemistry with the

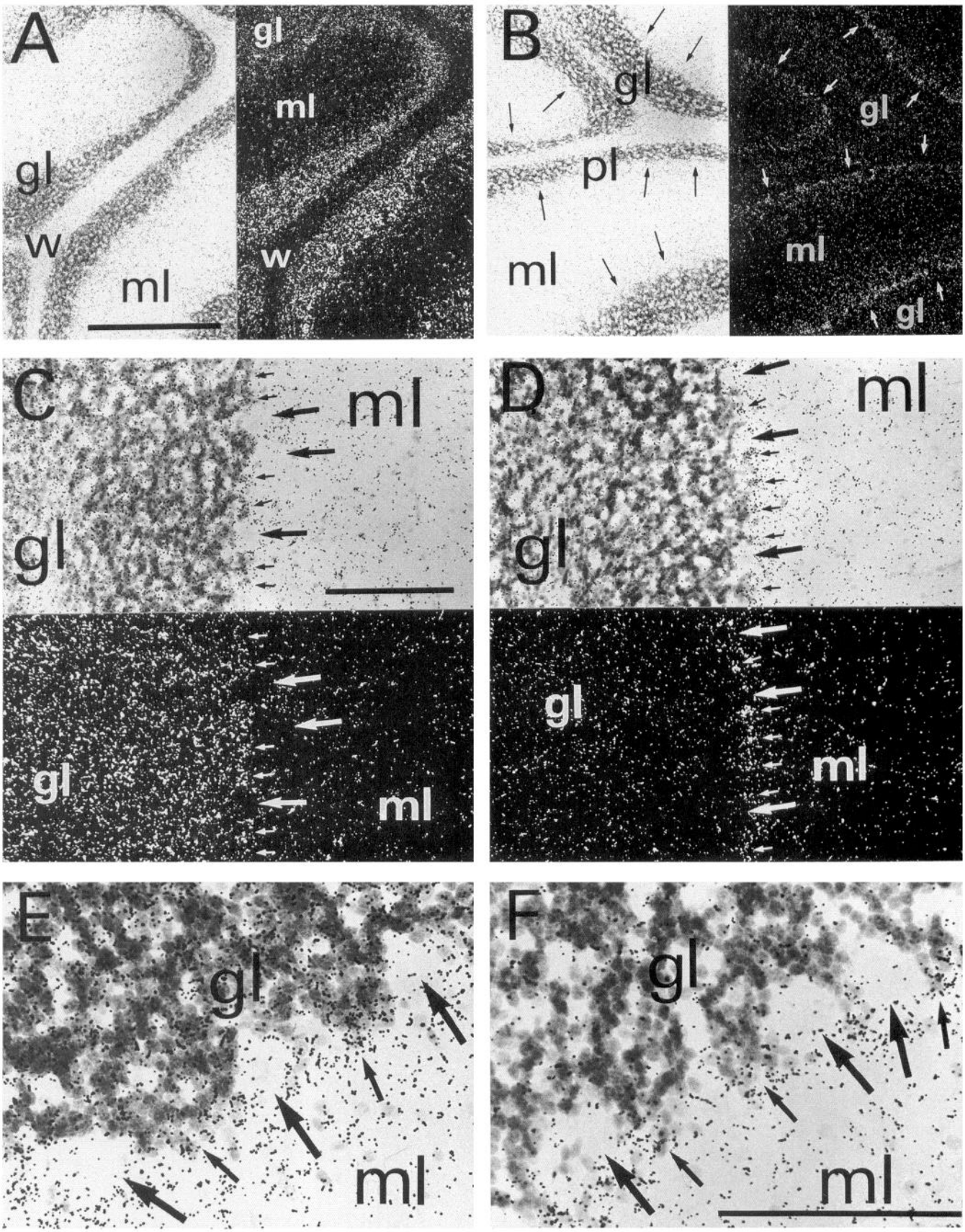

Figure 9. GluR-4c (*A, C, E*) and GluR-4 flip (*B, D, F*) transcripts are preferentially expressed in different cell types in the adult cerebellum. Low-power (*A, B*) bright-field and dark-field photomicrographs of adult cerebellum (P40) show that GluR-4c is found in both granule cell (*gl*) and

oligonucleotide probes used above showed different patterns of expression for GluR-4c and GluR-4 flip. The results obtained on cerebellar sagittal sections from rats at different developmental stages are shown in Figures 8–10. The general labeling pattern seen on a film with probe D (specific for the C-terminus region of the GluR-4c cDNA) (Fig. 8*A*) was virtually identical to that obtained with a GluR-4 pan-oligonucleotide probe (oligo A) (data not shown), indicating that a significant subset of GluR-4 transcripts in cerebellum is GluR-4c. In the adult (P40), both probes labeled the granular layer intensely and showed a gradient in the molecular layer that was high near the Purkinje cell layer and diminished near the pia. In stark contrast, probe B (specific for the GluR-4 flip module) resulted in a different pattern with no signal above background in the granular layer (Fig. 8*B*). Similarly, at earlier developmental stages (P8), the labeling pattern observed at low resolution consistently showed that granule cells were the major cell type expressing GluR-4c transcripts, whereas GluR flip was restricted to the Purkinje cell layer (Fig. 8*C,D*). Furthermore, it was already clear on film that GluR-4c, in contrast to GluR flip, is expressed by premigratory granule cells in the external germinal layer (Fig. 8*B,D*). Since the hybridization patterns obtained with the probes at P1 and P8, and at P20 and P40 were practically identical, we are only presenting data for P8 and P40.

The cellular distribution of transcripts derived from the GluR-4 gene was analyzed in sections 12 μm (Fig. 9) and 20 μm (Fig. 10) thick from adults (P20 and P40) or neonates (P1 and P8) after emulsion autoradiography. The higher resolution clearly showed that GluR-4c is expressed abundantly in granule neurons and Bergmann glial cell bodies (Fig. 9*A,C,E*). Furthermore, a gradient-like labeling was still observed in the molecular layer, which appeared to correspond to Bergmann glial processes. Labeling in the Purkinje cell layer, while definitely associated with Bergmann glial cell bodies, was not associated with the Purkinje cells (Fig. 9*C,E*). In striking contrast, the flip-specific probe hybridized exclusively to Bergmann glial cell bodies and processes, while granule cells did not show labeling above background (Fig. 9*B,D,F*). A sense probe (oligonucleotide F: 5′-GGGAGGAAACCTGGATTCCAAAGGCTATGGTGTAGCAACGCCAAGGG-3′, corresponds to position 2235), employed as a negative control, did not label the granular layer, the molecular layer, or the Purkinje cell layer and showed labeling that was slightly above background in the white matter (data not shown).

The developmental *in situ* studies confirmed the exclusive labeling of Bergmann glia for GluR flip (Fig. 10*B,D,F*) and clearly showed that premigratory granule cells express GluR-4c (Fig. 10*A,C,E*). Thus, premigratory granule neurons in the innermost region of the external germinal layer were labeled with the GluR-4c–specific probe, whereas cells in the proliferative zone adjacent to the pia were not labeled above background (Fig. 10*A,C,E*). In fact, in contrast to GluR-4 flip (Fig. 10*D,F*), GluR-4c–labeled cells were found in the incipient molecular layer leading to the already intensely labeled internal granular layer (Fig. 10*C,E*). As in adult, neither GluR-4c (Fig. 10*E*) nor GluR-4 flip (Fig. 10*F*) was expressed in Purkinje cells.

Discussion

General structural features of GluR-4c

We have isolated and characterized a cDNA clone from a rat cerebellar library that is highly homologous to GluR-4 flop isolated by Keinänen et al. (1990) and by Bettler et al. (1990) but contains a different module of 36 contiguous amino acids in the protein C-terminus domain. Stable mRNAs corresponding to the isolated GluR-4c flop cDNA could be identified in cerebellar tissue by using a specific oligonucleotide probe corresponding to the C-terminus domain. The existence of discrete bands on Northern blots that hybridize to the GluR-4c probe indicates that these transcripts exist as distinct, stable molecular species in the rat cerebellum. Direct molecular evidence for the existence of GluR-4c in cerebellum is the fact that we have isolated two additional independent cDNA clones of different sizes from our primary library that contain a C-terminus domain identical to the GluR-4c flop cDNA. Since one of these cDNAs was found to be a flip isoform, we can conclude that GluR-4 transcripts are differentially processed both at the flip/flop and C-terminal domains.

The GluR-4 transcripts do not derive from different genes that recently duplicated, because restriction mapping of rat genomic DNA on Southern blots, hybridized with an oligonucleotide spanning a region common to the 3′ end of all GluR-4 transcripts, yields a single band (data not shown). Although we have not directly demonstrated the mechanism used to generate different GluR-4c transcripts, our results strongly suggest that GluR subunits having different C-termini can be obtained either by differential splicing or the use of cryptic splice sites. From results obtained on the Southern blots using GluR-4 cDNA probes that extend sequences between the proposed M4 transmembrane domain and the C-terminus, it can be concluded that there must be an intron(s) present between M4 and the C-terminus. Consistent with this idea is the fact that the amino acid sequences in GluR-1–4 are extremely similar or identical up to a lysine residue located 14 residues downstream of M4 and then abruptly diverge significantly past this point (Keinänen et al., 1990), suggesting this site may function as an intron/exon boundary (see Figs. 1, 2).

Functional properties of GluR-4c

We have injected RNA synthesized from the GluR-4c cDNA in *Xenopus* oocytes (Sumikawa et al., 1984) and studied some of the pharmacological and functional properties of the expressed channels. This constitutes the first electrophysiological characterization of a GluR-4 receptor subunit. As previously shown for GluR-1–3 (Hollmann et al., 1989; Boulter et al., 1990; Keinänen et al., 1990; Nakanishi et al., 1990; Sommer et al., 1990), GluR-4c forms homo-oligomeric channels in oocytes that can be activated by glutamate, kainate, AMPA, and quisqualate. The EC_{50} of the kainate response (44 μM) is in the same

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Purkinje cell (*pl*) layers (*A*), whereas GluR-4 flip is restricted to the Purkinje cell layer (*B, arrows*). Higher-power (*C–F*) bright-field and dark-field views show that both GluR-4c (*C*) and GluR-4 flip (*D*) hybridization patterns show a sharp boundary (*arrows*) between the labeled Purkinje cell layer and the unlabeled molecular layer (*ml*); however, only GluR-4c labeled the granule cell layer. Note also that both isoforms are expressed in Bergmann glia (*small arrows*), but not in Purkinje cells (*large arrows*). This is even more evident at higher magnification (*E, F*). *W*, white matter. Scale bars: *A* and *B*, 0.5 mm (50×); *C* and *D*, 250 μm (90×); *E* and *F*, 50 μm (900×).

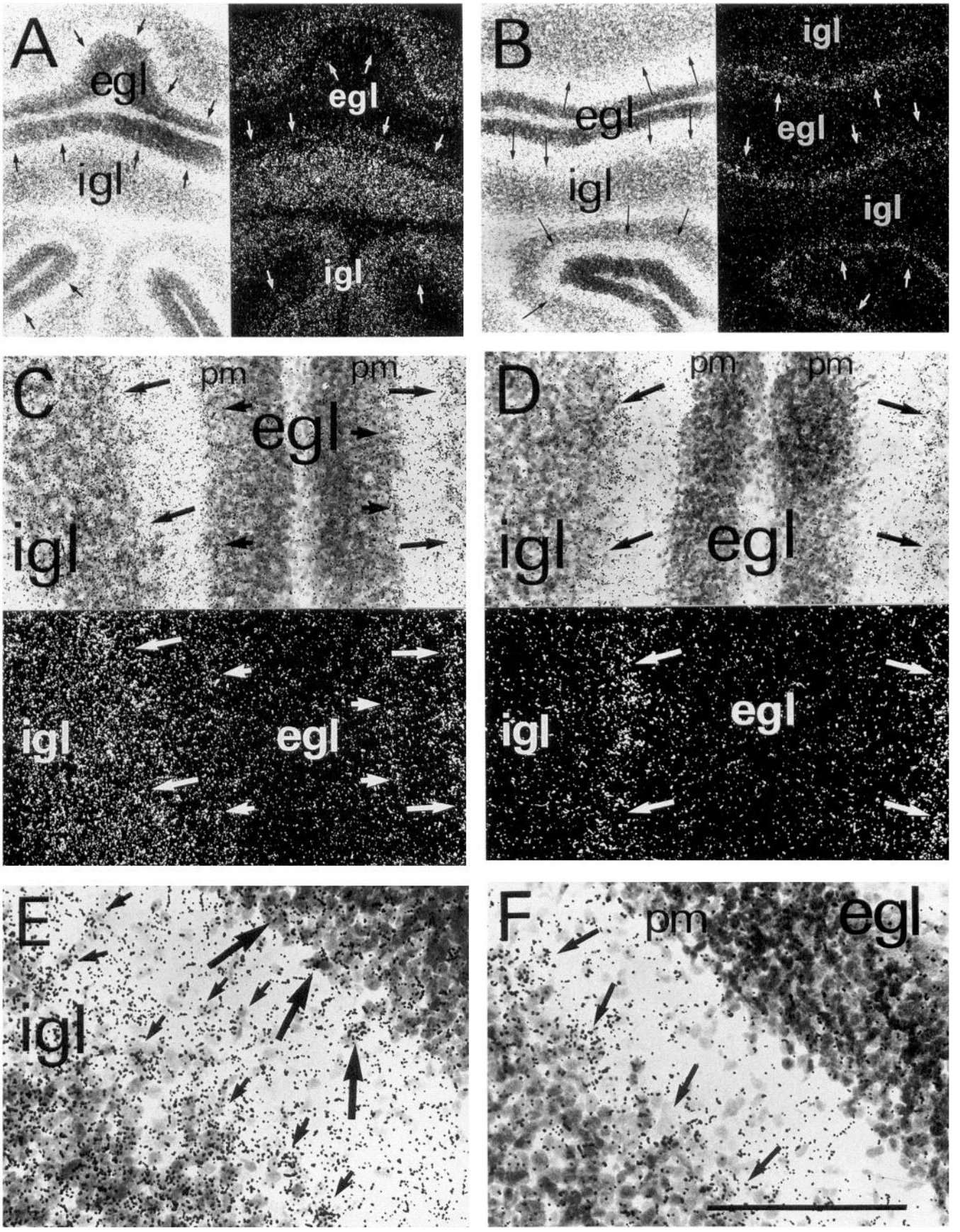

Figure 10. GluR-4c (*A, C, E*) and GluR-4 flip (*B, D, F*) have distinct developmental patterns in cerebellum. Low-power bright-field and dark-field paired photomicrographs of the developing cerebellum (P8) show that both the internal granule cell layer (*igl*) and the external germinal layer

order of magnitude of the EC_{50} for kainate found in oocytes injected with GluR-1 (32 μM; Nakanishi et al., 1990) or in cultured cerebellar granule cells (50 μM; V. Gallo, M. M. Usowicz, and S. G. Cull-Candy, unpublished observations), but lower than in cultured hippocampal neurons (143 μM; Patneau and Mayer, 1990) or in brain polyA+ RNA–injected oocytes (106 μM; Verdoorn and Dingledine, 1988). The EC_{50} for glutamate depolarizing responses (1.8 μM) in oocytes injected with GluR-4c was one order of magnitude lower than the EC_{50} for kainate, indicating that glutamate is a more potent agonist than kainate for GluR-4c channels. The inward currents elicited by kainate in GluR-4c injected oocytes were greatly reduced by the coapplication of quisqualate (data not shown), suggesting an interaction of these agonists at a common binding site located on the GluR-4c subunit. A recent kinetic study of kainate/quisqualate interactions in cultured hippocampal neurons has demonstrated that kainate, quisqualate, and AMPA interact competitively at the level of their receptors (Patneau and Mayer, 1991).

Boulter et al. (1990) and Nakanishi et al. (1990) have shown that the current–voltage relationship of kainate responses in oocytes injected with GluR-1, GluR-2, or GluR-3 displays a pronounced voltage dependence, that is, strong inward rectification. In oocytes injected with GluR-4c we also observed a nonlinear *I/V* relation for kainate responses with a pronounced inward rectification. This type of response is different from the linear *I/V* relation for kainate currents generated in either oocytes injected with cerebellar RNA (Randle et al., 1988) or in cultured cerebellar granule cells (Cull-Candy et al., 1988) and astrocytes (Usowicz et al., 1989). Therefore, GluR-4c may require additional subunits to form channels that behave similarly to the native receptor complex (Boulter et al., 1990; Nakanishi et al., 1990). In conclusion, these results show that the general pharmacological and functional properties of the GluR-4c homooligomeric channels are very similar to those of the other members of the kainate/AMPA receptor family, and hence further studies will be required to elucidate the different functional roles played by GluR-4 transcripts, including GluR-4c.

Complexity of GluR-4 gene transcripts

One of the aims of the present study was to determine which bands on the Northern blots corresponded to transcripts coding for the three variants (GluR-4c and GluR flip/flop) and to study their distribution in different cell types in the rat cerebellum. By using GluR-4c– and GluR flip/flop–specific oligonucleotides, we have found that each probe hybridizes to both the 6.2 and the 4.2 kb transcripts with approximately equal intensity but does not bind to the 3.0 kb band. The lower molecular weight transcript only hybridized with the pan-oligonucleotide probe corresponding to the N-terminal region of the GluR-4 subunits. In addition, the *in vitro*–synthesized transcripts from the GluR-4c cDNA clone were larger than the 3.0 kb band when analyzed on Northern blots (data not shown). These data strongly suggest that this RNA derives from the GluR-4 gene by yet a different form of processing resulting in a transcript missing sequences coding for the flip/flop and C-terminal domains.

This complex pattern of transcripts generated from a single GluR gene is not restricted to GluR-4. Northern blots probed with GluR-1 cDNA probes reveal most of the hybridization to a 5.2 kb band, but two minor bands at 3.9 and 3.2 kb are also observed (Hollmann et al., 1989). Multiple transcripts have also been reported for two kainate-binding protein cDNAs cloned from frog brain (Wada et al., 1989) and chick cerebellum (Gregor et al., 1989). Although functional channels have not been observed in oocytes injected with transcripts synthesized from the kainate–binding protein cDNAs, their sequences are closely related to the GluR genes described in mammals (Betz, 1990).

Cellular and developmental expression of GluR-4 transcripts in the cerebellum

The *in situ* hybridization studies showed that GluR-4c RNAs are highly expressed in cerebellar granule cells and in Bergmann glia, whereas GluR-4 flip is expressed only in Bergmann glial cells. These findings indicate that these products of GluR-4 gene processing are differentially expressed in cell populations of the cerebellar cortex that have a different developmental history (Altman, 1972a,b). Sommer et al. (1990) have shown that, in the hippocampus, GluR-2 flip is expressed only in CA3 cells, whereas GluR-2 flop is expressed only in CA1 and dentate gyrus neurons. Our results obtained for GluR-4 isoforms in the cerebellum provide, therefore, another interesting example of cell-specific processing for a glutamate receptor subunit in the CNS.

Granule cells are the most numerous cell type expressing GluR-4c in the cerebellum. In the rat cerebellar cortex these neurons proliferate postnatally in the external germinal layer, and after migrating to their final position, the internal granular layer, they receive an excitatory glutamatergic input from the mossy fiber terminals (Somogyi et al., 1986; D'Angelo et al., 1990). These developmental events occur between P1 and P20 (Altman 1972a,b), during the same time that we observed by Northern blot an increase in the levels of GluR-4c transcripts. The postnatal synchronous increase of the three cerebellar GluR-4 RNAs may result, therefore, from the maturation of granule neurons. These findings are also in agreement with previous binding and autoradiographical studies by Slevin and Coyle (1981) and Cambray-Deakin et al. (1990), which have shown that the density for binding sites of ^{3}H-kainate and ^{3}H-AMPA in the cerebellar cortex increases during granule cell maturation.

When do granule cells start expressing GluR-4c RNAs and what developmental events initiate their expression? Our *in situ* developmental studies interestingly show that granule neurons express messages for GluR-4c immediately before migrating to their final anatomical destination. Postmitotic granule cells la-

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(*egl*) are labeled with the GluR-4c–specific probe (*A*), but not with the flip-specific probe (*B*), further indicating that cells with different origins express different isoforms. *C* and *D*, Higher-power bright-field and dark-field photomicrographs show that GluR-4 flip–labeled cells are confined to the Purkinje cell layer (*D, large arrows*), whereas GluR-4c–labeled cells are present in both the internal granular layer and in the Purkinje cell layer, reflecting the finding in adult that both neurons and Bergmann glia are labeled already at P8. GluR-4c is also expressed in premigratory cells (*pm, small arrows*) in the external germinal layer, but not in the proliferative zone adjacent to the pia. This is more clearly shown in higher-resolution micrographs (Fig. 9*E, F*): note that GluR-4c–labeled cells (*E*) in the premigratory zone of the external germinal layer (*large arrows*) are labeled, as well as cells in the adjacent, incipient molecular layer leading to the internal granular layer (*small arrows*). In contrast, GluR-4 flip–labeled cells are only observed in the region of the internal granular layer adjacent to the molecular layer, supporting the idea that these are Bergmann glia. Scale bars: *A* and *B*, 0.5 mm (50×); *C* and *D*, 250 μm (90×); *E* and *F*, 50 μm (750×).

beled with the GluR-4c–specific probe were clearly demonstrated both in the premigratory zone of the external germinal layer and, as migrating cells, in the incipient molecular layer. These findings indicate that the expression of GluR-4c is either induced by highly localized signals in the premigratory zone of the external germinal layer or is regulated by an internal developmental clock set on the number of divisions of the granule neurons in the external germinal layer. Both hypotheses are compatible with the finding that granule cells dividing in the outermost part of the germinal layer were unlabeled with any of the probes for transcripts of the GluR-4 gene, as evidenced in tissue sections from P1 (not shown) or P8 cerebella.

Expression of GluR-4 transcripts in different cerebellar neural cells in culture

The results obtained *in vivo* prompted us to analyze the expression of GluR-4 transcripts in different types of cerebellar cells maintained in primary culture. The results obtained were in agreement with the findings *in vivo*. Cerebellar granule cells in culture express transcripts derived from the GluR-4 gene. The hybridization pattern on Northern blots obtained with RNA prepared from cultured granule neurons and cerebellar tissue were identical; bands of 6.2, 4.2, and 3.0 kb were observed. These transcripts were present *in vitro* as early as 2 hr after seeding. In this respect, granule neurons in culture are equivalent to postmitotic granule cells in the internal granular layer rather than to dividing granule cells of the external granular layer (see discussion above). This hypothesis is supported by the previous findings that cerebellar granule neurons do not divide in culture, but begin to grow neurites as early as 30–60 min after seeding (Thangnipon et al., 1983). Interestingly, no glutamate or kainate whole-cell current can be detected in dissociated cultured granule neurons before 4–5 d *in vitro* (Gallo, Usowicz, and Cull-Candy, unpublished observations). These observations suggest that a few days are necessary to assemble functional kainate/AMPA channels in the granule cell membrane, or that additional subunits expressed later during development need to combine with GluR-4 to form functional channels.

Northern blot analysis of RNA isolated from cerebellar astrocytes (type 1 and type 2; Raff et al., 1983; Levi et al., 1986) cultured *in vitro* revealed that the lower molecular weight transcript derived from the GluR-4 gene (3.0 kb) is missing in these cells, whereas the 6.2 and the 4.2 kb transcripts are expressed. The protein coded by these transcripts may assemble into functional receptors, since cultured astrocytes have been shown to express kainate- and quisqualate-activated channels (Bowman and Kimelberg, 1984; Usowicz et al., 1989) that open in the millisecond scale and possess subconductance states very similar to those described in neurons (Cull-Candy and Usowicz, 1987; Jahr and Stevens, 1987). The absence of the lower molecular weight transcript in astrocytes raises the possibility that the process generating the 3.0 kb transcript may be restricted to neurons, and that these mRNAs code for subunits that assemble to form receptors that differ in their structural components from glial glutamate receptors. In this respect, it is interesting that Cull-Candy et al. (1988) have also demonstrated the existence of channels with an estimated conductance of 140 fS in cerebellar granule cells; it is tempting to speculate that the 3.0 kb transcript, which increases in these cells during their maturation *in vitro*, encodes for a structural component of these channels.

Conclusions

Heterogeneity among the different ligand-gated channels such as those gated by ACh, glycine, GABA, and glutamate originates in great part from the large number of related genes that code for different receptor subunits (Betz, 1990). Furthermore, differential splicing adds another level of complexity because multiple subunit variants can be generated from a single gene. Alternative splicing of transcripts coding for neuronal nicotinic ACh (Goldman et al., 1987), glycine (Malosio et al., 1991), and glutamate receptor subunits (Sommer et al., 1990) has been observed. In addition, Sommer et al. (1990) have found that receptors formed by combinations of different GluR flip and flop proteins form channels with distinct functional properties. Thus far, we have not found that the C-terminal domain in the GluR-4c protein causes differences in the functional properties of homo-oligomeric channels expressed in oocytes. However, it is possible that specific combinations of GluR-4c with other GluR subunits are necessary to manifest changes in the functional properties of the receptor. The fact that the shorter C-terminal domains in proteins coded by three separate GluR genes have maintained up to 72% identity during evolution strongly suggests that this region is functionally important. An alternative role for the C-terminal domains of GluR subunits may be that they are involved in localizing or stabilizing the receptors to specific regions of the cell.

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