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The rat delta-1 and delta-2 subunits extend the excitatory amino acid receptor family

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We have characterized a second member (delta-2) of a new class of subunits for the ligand-gated excitatory amino acid receptor superfamily. The sequence of delta-2 exhibits an average identity of 25% and 18.5% to the non-NMDA and NMDA receptor subunits, respectively. The rat delta-2 gene is expressed predominantly in Purkinje cells of the cerebellum whereas only low levels of delta-1 transcripts are found in the adult brain. However, delta-1 gene expression undergoes a pronounced developmental peak, with particularly high mRNA levels in the caudate putamen of late embryonic/early postnatal stages.

Glutamate receptor; Rat brain; Purkinje cell

1. INTRODUCTION

The majority of fast excitatory transmission in the brain employs receptor channels activated by excitatory amino acids (EAAs). Traditionally, these have been classified on the basis of binding affinities of certain exogenous agonists as *N*-methyl-D-aspartate (NMDA), amino-3-hydroxy-5-methyl-isoxazole-4-propionate (AMPA) and high-affinity kainate sites [1]. The cloning of the first cDNA encoding a functional glutamate-gated ionotropic channel subunit [2] initiated an intense search for related polypeptides/genes (reviewed in [3,4]). It is now apparent, at least in mammalian species, that the three 'classical' sites are constructed combinatorially from members of a large family of polypeptides which function as channel subunits [3,4]. A high-affinity AMPA/low-affinity kainate series (GluR-A to -D) [2,5–8], a high-affinity kainate series KA-1 [9], KA-2 [10,11], GluR-5 [12,13], GluR-6 [14], GluR-7 [15,16] and the subunits that assemble into the NMDA-specific receptor channels i.e. NR1 [17] and NR2A, -2B and -2C [18–20] have been characterized. All of the cognate genes are differentially expressed in the rodent brain, suggesting that EAA signalling utilizes a diverse receptor repertoire.

One additional member of this superfamily, termed delta, has been isolated by homology screening of mouse brain cDNA libraries, but a functional profile was not determined [21]. Here, utilizing a similar approach, we show that the rat expresses at least 2 delta subunit genes, each characterized by a distinct expression profile in the developing and the mature rodent brain.

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2. MATERIALS AND METHODS

2.1. *Isolation of cloned delta-1 and delta-2 cDNAs*

Partial sequences of delta-1 and delta-2 cDNA were obtained from rat brain cDNA using the polymerase chain raction (PCR [22]) with degenerate oligonucleotide primers designed after largely conserved peptide sequences in ionotropic EAA receptor subunits [3,4]. The primers [5′-GCGAATTCTGGAA(C,T)GG(C,A)TGATGGG-(G,A,T,C)-GA-3′; 5′-GCGGTACCAA(A,G)GC(A,T,G)CCA(A,G)-(A,G)TT(A,T,G)GC(AT)GT(A,G)T-3′] constructed to peptides WNGMMGEI (60 residues NH_2-terminal to TM1) and YTANLA-AF(Tm3) produced the partial delta-1 sequence. The primers [5′-CGGAATTCTGCTGC(TA)A(GATC)GG(GATC)TT(TC)TG(CT)-AT(ATC)GA-3″] and [5′-CCGGTACCAC(GATC)GC(GATC)-AA(TC)CT(GATC)GC(GATC)GC(GATC)TT(CT)-3′] constructed to peptides CC(KY)GFLID and TANLAAF (TM3) yielded the partial delta-2 sequence. Full-length cDNAs were obtained by screening rat brain λgt10 cDNA libraries with ^{32}P-labeled PCR-generated DNA fragments as probes.

2.2. *Ligand binding studies and electrophysiology*

The delta-1 and -2 cDNAs were subcloned from the λ vector into a eukaryotic expression vector, and these recombinant vectors were used to transfect HEK-293 cells [5,13]. Membranes were prepared for ligand binding as described [13]. Electrophysiological recordings were performed by patch-clamp techniques in the whole-cell configuration from single transfected cells [5,13]. Agonists (100–300 μM) were dissolved in normal rat Ringer solution and applied rapidly via a Piezo-driven double-barreled pipette [5,13].

2.3. *In situ hybridization*

In situ hybridization was performed with oligonucleotides [23]. The oligonucleotides were delta-1: 5′-GAAGCTGAGGGCCGAGGCT-GGGTGGCACTCTGAGACCTTACAGCC-3′ antisense of delta-1 peptide sequence QAVRSQSATQPRPSA between TM1 and TM2,

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delta-2: 5′-GTCATTGATCCCATTTGCAACCGTGGGGGAT-TAAGCCAGTTCAA-3′, antisense of delta-2 peptide WLNPPRL-QMGSMT between TM1 and TM2, and 5′-GAACCACACCTCG-CATGTTATTCTCCAGTTTCTTGTCTGTCAGT-3′, antisense of delta-2 sequence TDKKLENNMRGVV in the extracellular domain. Probes were labelled with terminal transferase (Boehringer, Mannheim), using the β-emitting radionucleotide [α-^{33}P]dATP (1,825 Ci/mmol, a gift from NEN Dupont [24]). A typical labelling reaction utilized 0.3 pmol oligonucleotide and 8 pmol [α-^{33}P]dATP. Unincorporated nucleotides were removed by chromatography on Bio-Spin 6 columns (Bio-Rad). Average specific activity obtained was 33.3×10^6 dpm/pmol oligonucleotide. Probes were used at a concentration of 1 pg/ml and hybridized at 42°C for 12 h in minimalist buffer, containing only 50% formamide/4× SSC/10% dextran sulphate. After hybridization sections were washed in 1× SSC at 60°C. Exposure time was 5 days on Kodak XAR-5 film and 3 weeks in Ilford K-5 emulsion. Signal specificity was confirmed on parallel sections by the use of a 200-fold excess of unlabelled oligonucleotide competing with labelled probe. The specificity of the delta-2 signal was further confirmed by the use of the two independent probes. Identical results were obtained with ^{35}S-labelled probes.

3. RESULTS AND DISCUSSION

As part of our efforts to fully define the ionotropic EAA receptor subunit family, PCR [22] with degenerate primers on rat brain cDNA was employed to obtain 2 novel cDNAs that encoded predicted proteins with only 17–28% homology to other family members. The first of these cDNAs encoded the rat homolog of the mouse delta-1 subunit [21]. The other is related but distinct, hence our designation of delta-2 (Fig. 1).

The delta-1 and -2 polypeptides, as predicted from the cDNA sequences, are approximately 1,000 residues long. The members of the delta subfamily show a signal peptide, a long putative extracellular domain containing consensus *N*-glycosylation sites, and hydrophobic regions which are likely to be transmembrane domains. The amino acid sequence identity between the 2 delta subunits is 56%, whereas the identity exhibited with

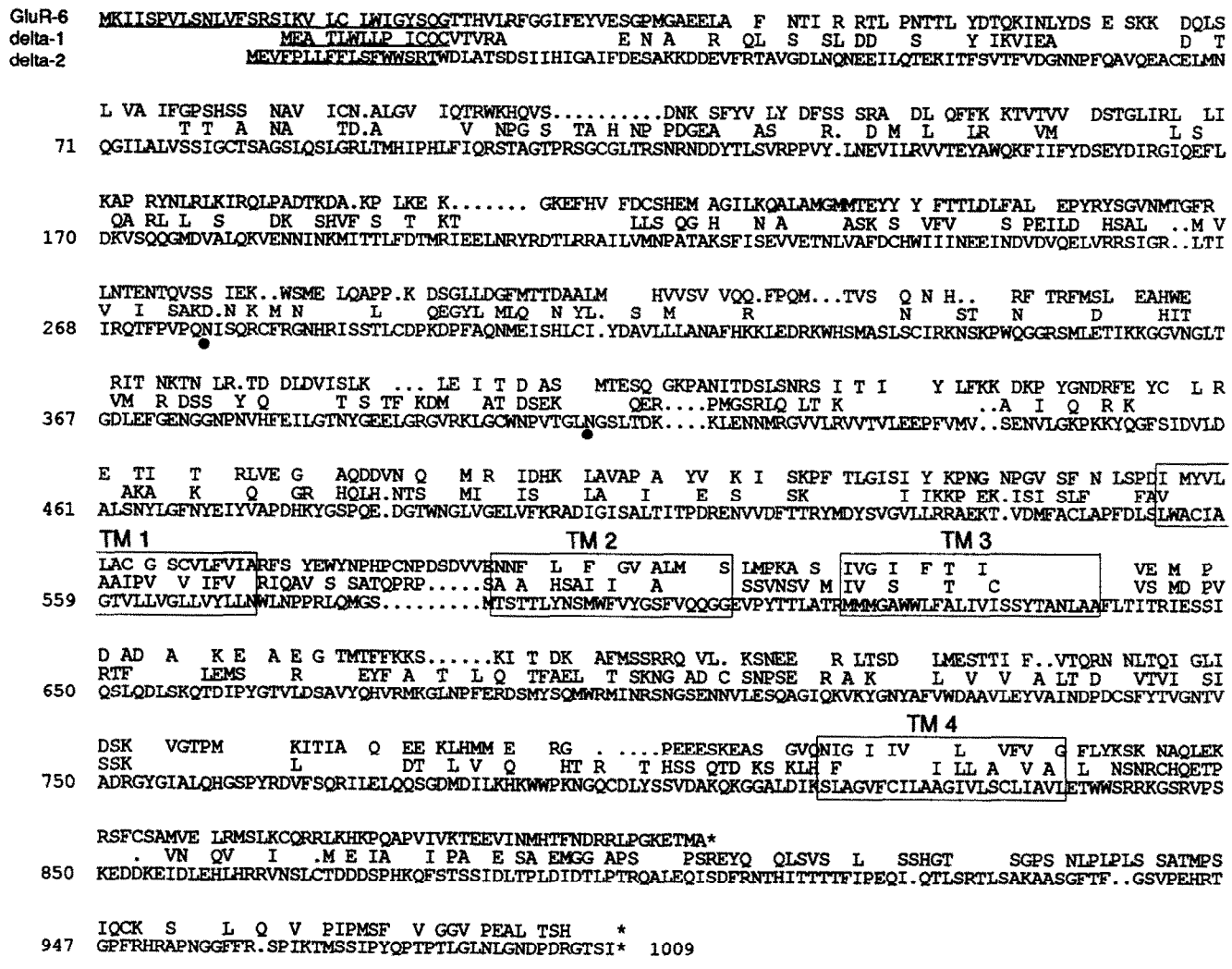

Fig. 1. Sequence alignment of the rat delta-1, delta-2 and GluR-6 polypeptides. At each position, delta-1 and GluR-6 sequences are listed only when they differ from delta-2. Sequences are numbered starting with their predicted mature N-termini and signal sequences are underlined. The putative transmembrane regions TM1–4 are boxed. *N*-Linked glycosylation sites (NxS/T, xP) in the predicted extracellular domain of the three subunits are indicated by filled circles. The rat delta-1 and delta-2 nucleotide sequences have been deposited in the EMBL Genbank database under accession numbers Z17238 and Z17239, respectively.

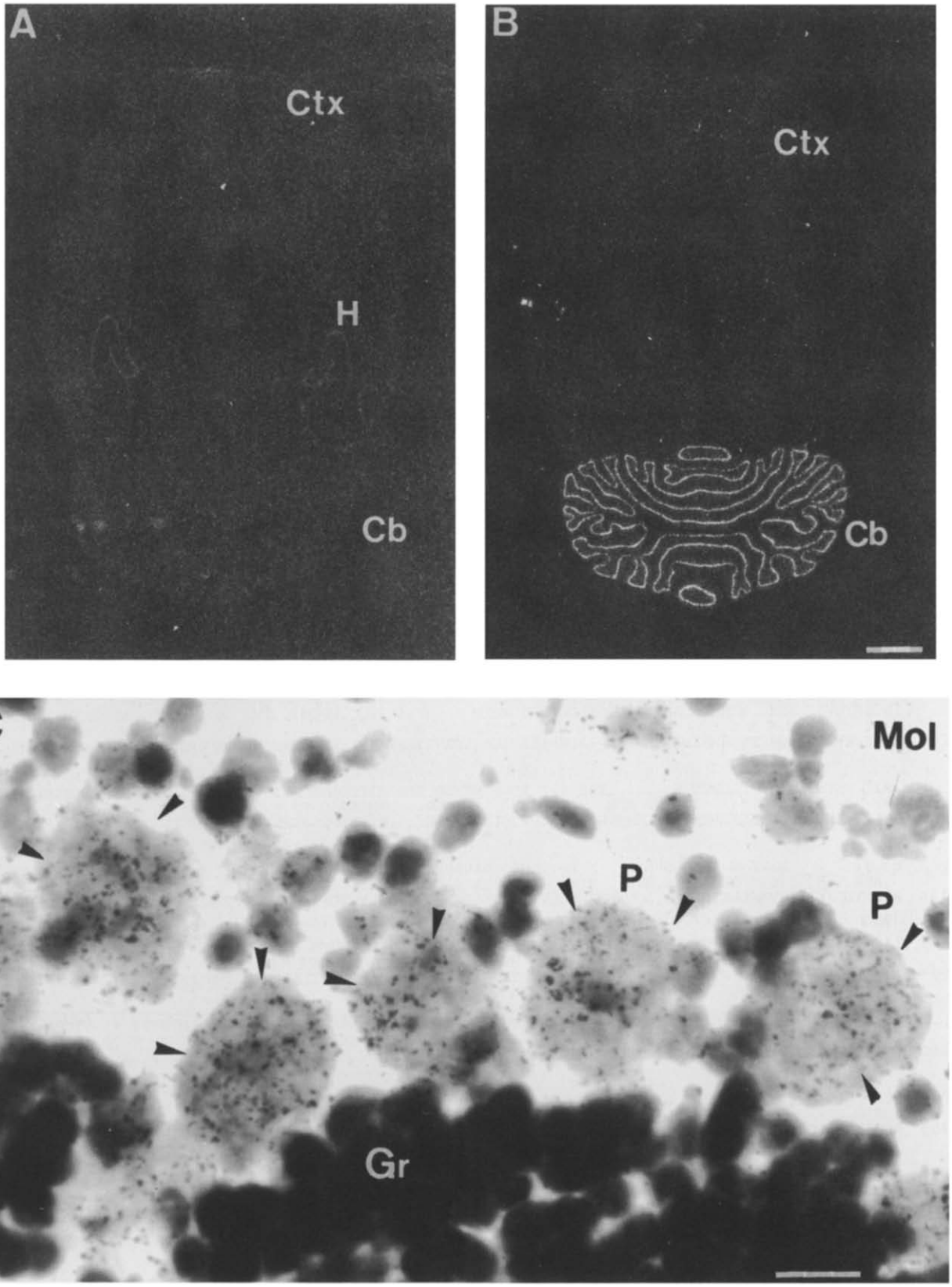

Fig. 2. X-ray film autoradiographs showing distribution of delta-1 (A) and delta-2 (B) mRNAs in horizontal sections of the adult rat brain as assessed by in situ hybridization with ^{33}P-labeled oligonucleotide probes. Cb, cerebellum; Ctx, cortex; H, hippocampus; bar, 3.5 mm. (C) Localization of delta-2 mRNA in Purkinje cells of the cerebellum with ^{33}P-labelled probe. High-power bright field optics. Gr, granule cells; Mol, molecular layer; P, Purkinje cell layer; arrowheads mark the labelled Purkinje cells. Bar = 28 mm.

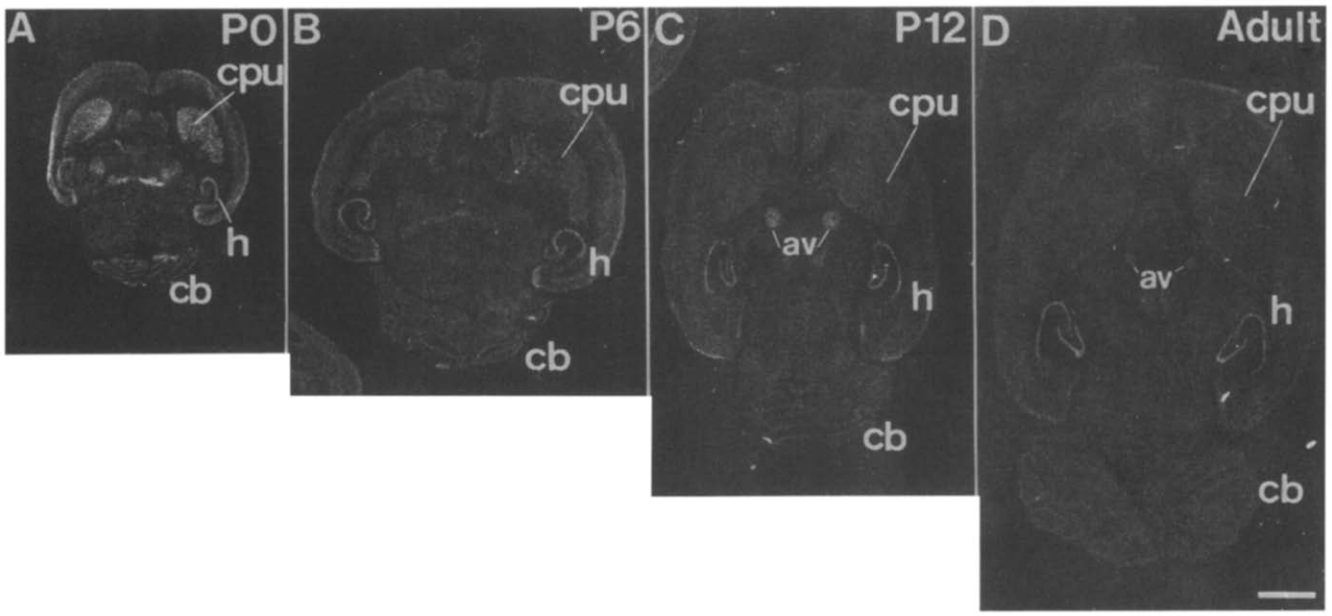

Fig. 3. Postnatal developmental expression of the delta-1 gene as assessed by in situ hybridization using a ^{35}S-labelled probe on horizontal sections. av, anteroventral thalamic nucleus; cb, cerebellum; cpu, caudate putamen; h, hippocampus. P0 is the day of birth. All images result from the same exposure time and are printed to the same scale, thus reflecting the increase in brain size with age. Bar = 3.3 mm.

members of the non-NMDA and NMDA receptor subunit subfamilies is only 22–28% and 17–20%, respectively. No significant higher identity is observed with any particular EAA receptor subfamily, although in the putative channel forming TM2 region, identity is closest to the non-NMDA subunits. This sequence property sets the delta subfamily apart from the previously characterized EAA receptors, and suggests that this new subfamily could exhibit a different ligand-binding profile.

We next examined the expression of the delta genes in the adult rat brain by in situ hybridization using probes labelled with the β-emitting radionucleotide [α-^{33}P]dAMP [24] recently made commercially available. The delta-1 probe gave a weakly diffuse but specific autoradiographic signal, with highest levels present in the pyramidal and dentate granule cell layers of the hippocampus (Figs. 2A and 3D). The pattern remained the same, even after longer exposure times or when ^{35}S-labelled probes were used (Fig. 3D). Hence, in our experience, the delta-1 mRNA is the least abundant EAA receptor transcript examined to date. In contrast, the delta-2 gene is expressed at high levels in the cerebellar Purkinje cell layer (Fig. 2B), with virtually no signal present in the forebrain. To differentiate between labelling of Purkinje cells and Bergmann glia, both cell types being present in the same layer of the cerebellum, emulsion studies were performed. Use of high power brightfield optics shows that the delta-2 specific signal originates from the Purkinje cells, which are densely decorated with silver grains (Fig. 2C). No signal was observed over the granule cells (not shown). Examination of other brain areas in coronal planes of section showed that there was also some weak but specific delta-2 gene expression in the outer layers of the cingulate cortex and in the dentate granule cells of the hippocampus (not shown). These results demonstrate the feasibility of using ^{33}P-labelled probes for in situ hybridization with both X-ray film and emulsion applications. However, the ^{35}S-tagged probes gave slightly better spatial resolution.

Although delta-1 gene expression is low in the adult brain, mRNA levels of this subunit are much higher in younger animals (Fig. 3). For example, at the day of birth (P0), the caudate putamen contains elevated levels of delta-1 mRNA (Fig 3A), which then gradually decline to those of the adult (Fig. 3D). Similarly, at P12, delta-1 expression is higher in the anteroventral (av) thalamic nucleus (Fig. 3C) relative to that of the adult (Fig. 3D). In contrast, the amount of hippocampal delta-1 mRNA remains essentially unchanged throughout postnatal periods. The timing of expression of the delta-1 gene may indicate a role in development (e.g. neurite extension, differentiation). With respect to the delta-1 gene expression no marked developmental changes were noted.

To determine the possible functional characteristics of the delta subunits, expression vectors containing the delta cDNAs were transfected into HEK-293 cells. Cells were then patch-clamped or membranes harvested for ligand-binding analysis. However, no specific binding was detected with [^{3}H]AMPA and [^{3}H]kainate, using either delta-1 or delta-2 homomeric expression. Similarly, glutamate (300 mM), kainate (300 mM) and

AMPA (100 mM) failed to activate whole-cell currents in cells expressing the homomeric delta subunits.

Because of its prominent expression in Purkinje cells, the delta-2 subunit was expressed in HEK 293 cells with representatives of other subunit classes whose mRNA/proteins are also found in Purkinje cells; i.e. GluR-A, -B, -C [5,25,26], GluR-5 [12,27], KA-1 [9], and NR-1 [17]. The whole-cell currents and I–V curves that resulted from applying glutamate (100 or 300 mM) and kainate (300 mM) onto cells co-expressing the delta-2 protein with the GluR-B or GluR-5 subunits showed no deviation from GluR-B or GluR-5 expression alone [13]. A delta-2/KA-1 and delta-1/GluR-7 combination also gave no kainate- or glutamate-evoked current responses. For the NMDA-responsive subunits, delta-2/NR-1, delta-2/NR-2A and delta-2/NR-2C combinations likewise gave no response to any agonist tested. Finally, all of the above listed combinations failed to give currents with the 2 sulphur-containing amino acid neurotransmitter candidates L-homocysteic acid and L-cysteinesulfinic acid [28]. However, clear responses to these two ligands were obtained using NR-1/NR-2A heteromeric assemblies (not shown). To ascertain that these negative findings were not due to any defects in the cloned delta cDNAs, in vitro transcribed cRNA was translated in a reticulolysate system. Both the delta-1 and delta-2 cRNAs yielded proteins of the expected size (appr. 90 kDa, not shown).

Hence, although members of the ligand-gated EAA receptor subunit family, the two delta polypeptides remain 'orphan' subunits. Failure to obtain a channel response to applied ligand is a frequently observed feature of putative EAA receptor subunits expressed from cDNAs. Similar situations apply to the KA-1 [9], KA-2 [10], GluR-7 [15,16], chick [29], and frog [30] kainate binding proteins, although in contrast to the delta subunits, at least 4 of these other proteins bind ligands when expressed in homomeric configurations. Furthermore, the KA subunits form ligand-gated channels only when assembled with the GluR-5 or -6 subunits [10,11]. This should provide an impetus to search for missing partners for the delta subunits.

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