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20. Plasmid pTM200 was provided by G. Cornelis. The coding sequence for YOP 51 was cloned by polymerase chain reaction with two primers. The sequences of the 5′- and 3′-primers are 5′-GTCGGATCCAGAATTCGCATGAACTTATCAATTAAG-3′ and 5′-GTCGGATCCTGAATTCGAATAAATATTTACATTAGC-3′, respectively. The 1.4-kb PCR product was digested with Eco RI and subcloned into the Eco RI site of vector pT7-7 to construct pT7–YOP 51. The amino acid sequence encoded by the plasmid consists of the first five amino acids of T7 gene 10 protein followed by the complete coding sequence of YOP 51. Plasmid pT7-YOP-PTP was constructed by the following steps: the PCR product was digested with Xba I and then blunt-ended by treatment with Klenow fragment, followed by digestion of Bam HI. This fragment encodes amino acid residues 154–468 of YOP 51. The PTPase domain of YOP 51 was expressed in the correct reading frame following directional ligated into pT7-7 vector between a filled-in Eco RI site and a Bam HI site.
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23. The YOP 51 coding sequence was subcloned from pT7–YOP 51 into M13MP18 for site-directed mutagenesis. Cys^{403} was mutated to either Ala or Ser with the in vitro mutagenesis kit (Amersham). The mutated DNA was subcloned into pT7-7 for expression.
24. Supported by grants from the NIH (National Institute of Diabetes and Digestive and Kidney Diseases 18849) and the Walther Cancer Institute. We would like to thank G. R. Cornelis, Université Catholique de Louvain, Brussels, Belgium, for plasmid pTM 200; J. D. Corbin, Vanderbilt University, School of Medicine, for the catalytic subunit of protein kinase A; K.-H. Kim (Purdue University) for the purified rat insulin receptor; H. Qiu for technical assistance; and the Purdue AIDS Center Grant (A127713) for support of the computer facilities. Special thanks to T. Woodford, D. Pot, and E. Remboutsika for helpful discussions and reading the manuscript. This is journal paper number 12486 from the Purdue University Agricultural Experimentation Station.

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Table 1. Agonist-evoked current amplitudes of glutamate receptors expressed in vitro. Numbers represent average amplitudes (mean ± SEM) of inward currents (pA) evoked by agonist at a holding potential of −60 mV. Values of *n* are in parentheses below.

Receptors	Agonists (100 μM)	
	L-Glu	Kainate
GluR-A	32 ± 9 (9)	46 ± 13 (8)
GluR-B	8 ± 6 (4)	25 ± 12 (7)
GluR-C	7 ± 3 (6)	30 ± 7 (6)
GluR-D	22 ± 6 (6)	41 ± 11 (6)
GluR-A, -B	68 ± 16 (15)	216 ± 32 (17)
GluR-B, -D	13 ± 3 (4)	160 ± 25 (4)
GluR-A, -B, -C, -D	16 ± 17 (6)	107 ± 32 (6)

A Family of AMPA-Selective Glutamate Receptors

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Four cloned cDNAs encoding 900–amino acid putative glutamate receptors with approximately 70 percent sequence identity were isolated from a rat brain cDNA library. In situ hybridization revealed differential expression patterns of the cognate mRNAs throughout the brain. Functional expression of the cDNAs in cultured mammalian cells generated receptors displaying α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)–selective binding pharmacology (AMPA = quisqualate>glutamate>kainate) as well as cation channels gated by glutamate, AMPA, and kainate and blocked by 6,7-dinitroquinoxaline-2,3-dione (CNQX).

MOST NEURONS IN THE CENTRAL nervous system are excited by glutamate. The postsynaptic actions of this neurotransmitter are mediated by at least three major, pharmacologically distinct classes of ionotropic receptors as well as by a metabotropic receptor at which glutamate modulates the release of intracellular Ca^{2+} (*1*). The ionotropic receptors contain glutamate-gated cation channels and are named according to their selective agonists as the NMDA (*N*-methyl-D-aspartate), AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), and kainate receptors. The pharmacological and electrophysiological properties of NMDA receptors are well studied (*1*). However, because there are no selective antagonists, the characterization of the non-NMDA receptors is less advanced, and it has been difficult to distinguish kainate and AMPA receptors (*2*). Non-NMDA receptors are the major mediators of fast glutamatergic neurotransmission and may contribute to synaptic plasticity (*3*). Glutamate-induced neurotoxicity, a pathological process of widespread clinical importance also involves these receptors (*4*). Hence, the key role played by glutamate-operated channels in virtually all physiological and many abnormal brain functions accounts for the ever increasing efforts to elucidate their molecular design.

Recently, Hollman and co-workers characterized a molecule designated GluR-Kl (*5*), the sequence of which was similar to kainate binding proteins of chick and frog (*6*). To study the diversity of this family and the pharmacology of its members, we isolated cDNAs encoding several sequence-related novel receptors. We obtained a result that was unexpected from the assignment of GluR-Kl as a kainate receptor subtype (*5*): these polypeptides made up a family of AMPA receptors as indicated by their functional expression and distribution of their mRNAs in rat brain.

Cloned cDNAs encoding the receptors were obtained via polymerase chain reaction (PCR)–mediated DNA amplification by using published sequences (*5, 6*) for primer design and by the subsequent screening of cDNA libraries constructed from rat brain mRNA. Four molecular species, designated GluR-A to -D, were fully characterized with GluR-A showing 100% sequence identity to GluR-Kl. The four predicted polypeptide sequences (Fig. 1), each approximately 900 amino acids in length including a signal sequence, revealed upon pairwise comparisons overall similarities between 70% (GluR-A versus -B) and 73% (GluR-B versus -C). Similarities dropped to between 56% (GluR-A versus -D) and 63% (GluR-C versus -D) when comparison was restricted to the NH_2-terminal 470 amino acids of the mature polypeptides. This region contained the highest number of substitutions in the four receptors but specified the only con-

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served NH_2-linked glycosylation sites, in keeping with a predicted extracellular location. The COOH-terminal half, except the last 35 (GluR-B, -C) to 70 (GluR-A, -D) residues of the receptors, was extremely conserved, in marked contrast to that of the subunits of the nicotinic acetylcholine and γ-aminobutyric acid A ($GABA_A$) receptor families (7), which show high sequence diversity within this region. The conspicuous lack of sequence homology between the glutamate receptors and members of other ligand-gated ion channels (7), as well as the large difference in size, suggest a very distant or no evolutionary relationship.

Hence, the transmembrane topology of the glutamate receptors, based on four membrane-spanning regions (TM1 to TM4) and adopted here for simplicity (Fig. 1), is largely conjectural. In fact, other topologies for the COOH-terminal half of the receptors are compatible with the predicted polypeptide sequences, as illustrated in part by the incongruent assignment of transmembrane segments in sequence-related molecules (*5, 6*). However, in accordance with the cationic conductance of glutamate-operated channels, regions expected to be part of the inner and outer channel mouth reveal a preponderance of negatively charged residues. In particular, the loop connecting TM1 and TM2 carries a net negative charge, and TM2 is flanked by two negatively charged residues that may contribute to the charge rings determining channel conductance in the functional receptor (*8*). Moreover, the putative intracellular domain contains consensus phosphorylation sites for Ca^{2+}-calmodulin–dependent protein kinase type II and protein kinase C. These enzymes are candidates for mediation of the induction and maintenance of long-term potentiation (*3, 9*).

To study their functional activity, the four receptors were transiently expressed in cultured mammalian cells (*10*). With patch-clamp techniques (*11*), functional ion channels could be measured (*12*) upon expression of each of the four receptors. Inward currents (holding potential, −60 mV) activated by L-glutamate and kainate were observed in cells expressing single polypeptides as well as the combinations GluR-A and -B, GluR-B and -D, and all four receptors (Table 1 and Fig. 2). Preliminary evidence suggests that these channels have small unitary conductances (≤1 pS) and, hence, the whole cell currents reflect high channel numbers. Notably larger currents were mediated by the receptor combinations, indi-

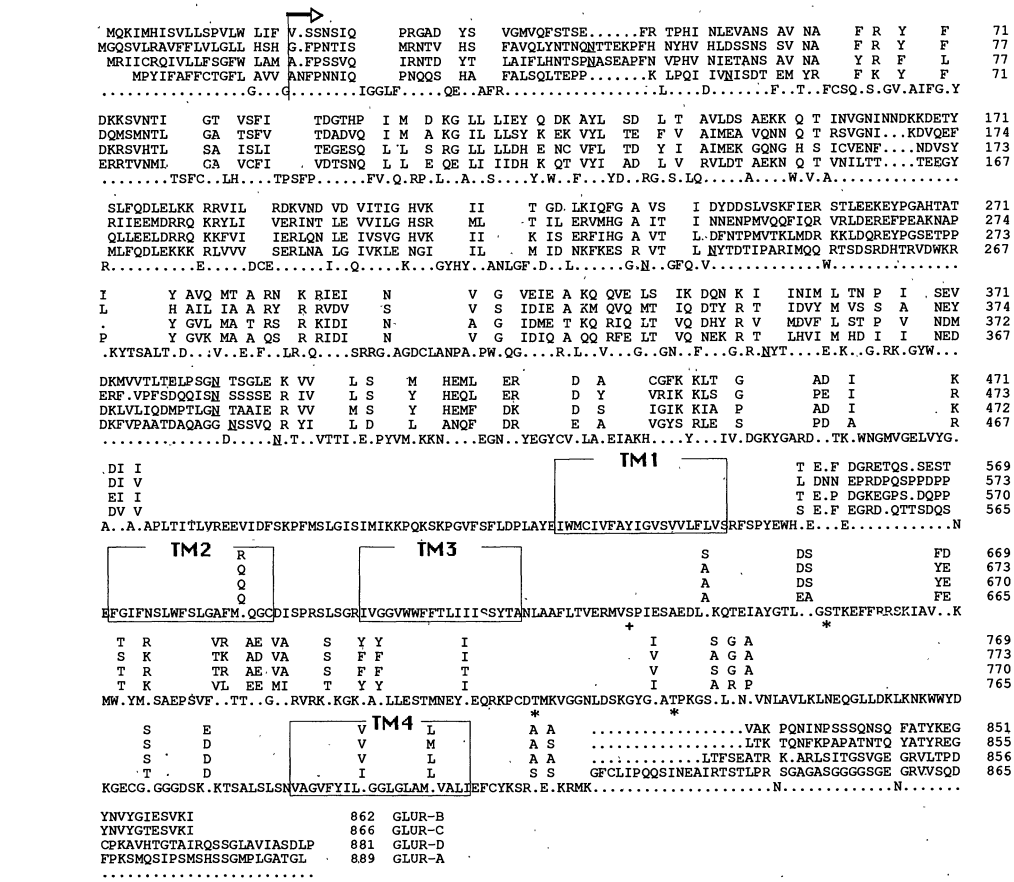


Fig. 1. Comparison of polypeptide sequences encoded by four putative glutamate receptor cDNAs. Sequences presented in single-letter code (*25*) and numbered on the right in the order of GluR-B, -C, -D, -A. Only differences from a consensus sequence are listed. GluR-A is identical to GluR-Kl (*5*). Arrow indicates the predicted mature NH_2-termini. Consensus NH_2-linked glycosylation sites are underlined, putative transmembrane regions are boxed, and consensus phosphorylation sites are indicated by + (Ca^{2+}-calmodulin–dependent protein kinase II) and * (protein kinase C). Two degenerate oligonucleotide primers designed to peptide sequences conserved in Glu-R-Kl (*5*) and two kainate binding proteins (*6*) were used to amplify homologous sequences from rat brain cDNA by PCR (*26*). The amplified DNA (≈520 bp) was excised from the gel and subcloned into M13 vectors for sequence analysis, taking advantage of the restriction endonuclease sites present in the 5′ ends of the primers. Cloned full-length cDNAs having sequences identified from the PCR products as encoding putative glutamate receptors were obtained by screening rat brain cDNA libraries (insert size >3.5 kb) constructed in λ ZAP II (Stratagene, CA) and λgt10 vectors, with ^{32}P-labeled PCR fragments as probes. The complete nucleotide sequences are deposited in the GenBank-EMBL database, accession numbers M36418 (GluR-A), M36419 (GluR-B), M36420 (GluR-C), M36421 (GluR-D).

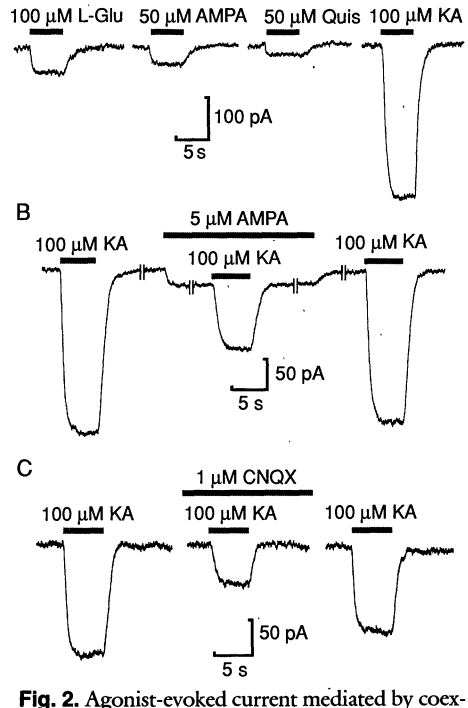


Fig. 2. Agonist-evoked current mediated by coexpressed GluR-A and -B receptors. (**A**) Comparison of inward currents at −60 mV activated by 5-s applications (denoted by bars) of 100 μM L-glutamate (L-Glu), 50 μM AMPA, 50 μM quisqualate (Quis), and 100 μM kainate (KA) to the same cell. (**B**) Interaction between AMPA and kainate. Traces show inward current activated by 100 μM kainate in the presence and absence of 5 μM AMPA. AMPA alone induced a small current and markedly reduced the kainate-evoked current when applied before AMPA. The currents evoked by AMPA and kainate were not additive, suggesting that at 5 μM AMPA bound to a large fraction of the available receptors without activating them. The effect was reversible as shown in the right trace. (**C**) Reversible block of kainate before, during, and after perfusion of CNQX. The cell was bathed in CNQX for 3 min before kainate plus CNQX was applied. CNQX alone produced no current (n = 6). The right trace was obtained approximately 5 min after wash with control extracellular solution.

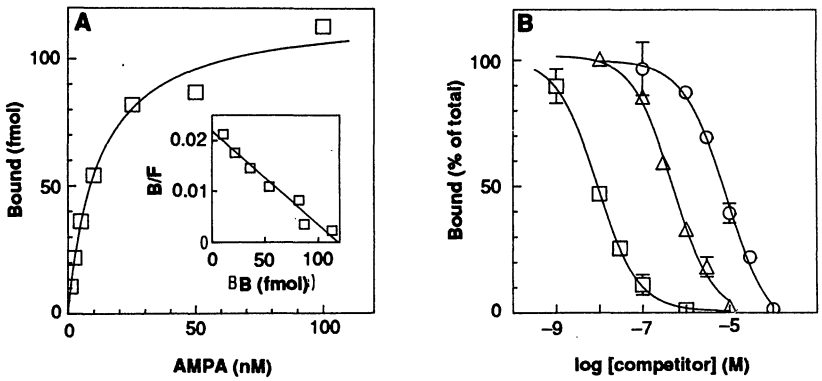


Fig. 3. Binding of [^{3}H]AMPA to GluR-B expressing cells. (**A**) Saturation binding isotherm and Scatchard plot (inset). Aliquots of homogenate of cells transiently expressing GluR-B were incubated in triplicate in 0.5 ml containing 30 mM tris-HCl, pH 7.2, 2.5 mM $CaCl_2$, 100 mM potassium thiocyanate with 1 to 100 nM [^{3}H]AMPA (29.2 Ci/mmol; New England Nuclear) for 30 min at 0°C, followed by rapid filtration through GF/C filters (*14*). Nonspecific binding was obtained by including 1 mM glutamate in the binding assay. The calculated K_d value is 11 nM; the maximum binding (B_{max}) corresponds to 40,000 sites per cell. (**B**) Displacement of [^{3}H]AMPA binding by unlabeled quisqualate (□), glutamate (△), and kainate (○). Binding was performed as above but with 5 nM [^{3}H]AMPA in the presence of increasing concentrations of unlabeled competitor. Results are expressed as percentage of maximal specific binding, obtained in the absence of competitor. The IC_{50} values for quisqualate, glutamate, and kainate are 9 nM, 490 nM, and 9 μM, respectively. The experiment was performed three times with essentially identical results. NMDA (50 μM) did not affect [^{3}H]AMPA binding.

cating that hetero-oligomeric receptors may assemble more efficiently or differ in their channel properties from homo-oligomeric receptors.

Further electrophysiological tests on coexpressed GluR-A and -B receptors showed activation by L-glutamate, quisqualate, AMPA, and kainate (Fig. 2A). The average amplitudes of agonist-evoked inward currents were (mean ± SEM, n = 7) 68 ± 16 pA for 100 μM L-glutamate, 16 ± 3 pA for 50 μM quisqualate, and 200 ± 55 pA for 100 μM kainate. Differences in affinity could account for the variation in current amplitude among these agonists, implying that kainate has the highest affinity for the receptor. However, an alternative explanation is that L-glutamate, quisqualate, and AMPA may function as partial agonists at this receptor with kainate a full agonist. Consistent with this latter possibility, when kainate (100 μM) was applied in the presence of AMPA (5 μM) the amplitude of kainate-evoked current (Fig. 2B) was reduced to 40 ± 2% of control (*13*) (n = 4). Furthermore, currents were potently blocked by the non-NMDA receptor antagonist CNQX (6,7-dinitroquinoxaline-2,3-dione) (*14*). At 1 μM, CNQX reduced the currents evoked by 100 μM kainate to 39 ± 3% of control (n = 6) (Fig. 2C). These results extend those reported for the GluR-K1 receptor expressed in *Xenopus* oocytes (*5*). Indeed, the pharmacological properties of the coexpressed GluR-A and -B receptors resemble those of the non-NMDA receptor expressed in oocytes from brain mRNA (*15*) and of neuronal non-NMDA receptors (*16*). By demonstrating AMPA sensitivity, our results suggest that the four receptors belong to the AMPA receptor family.

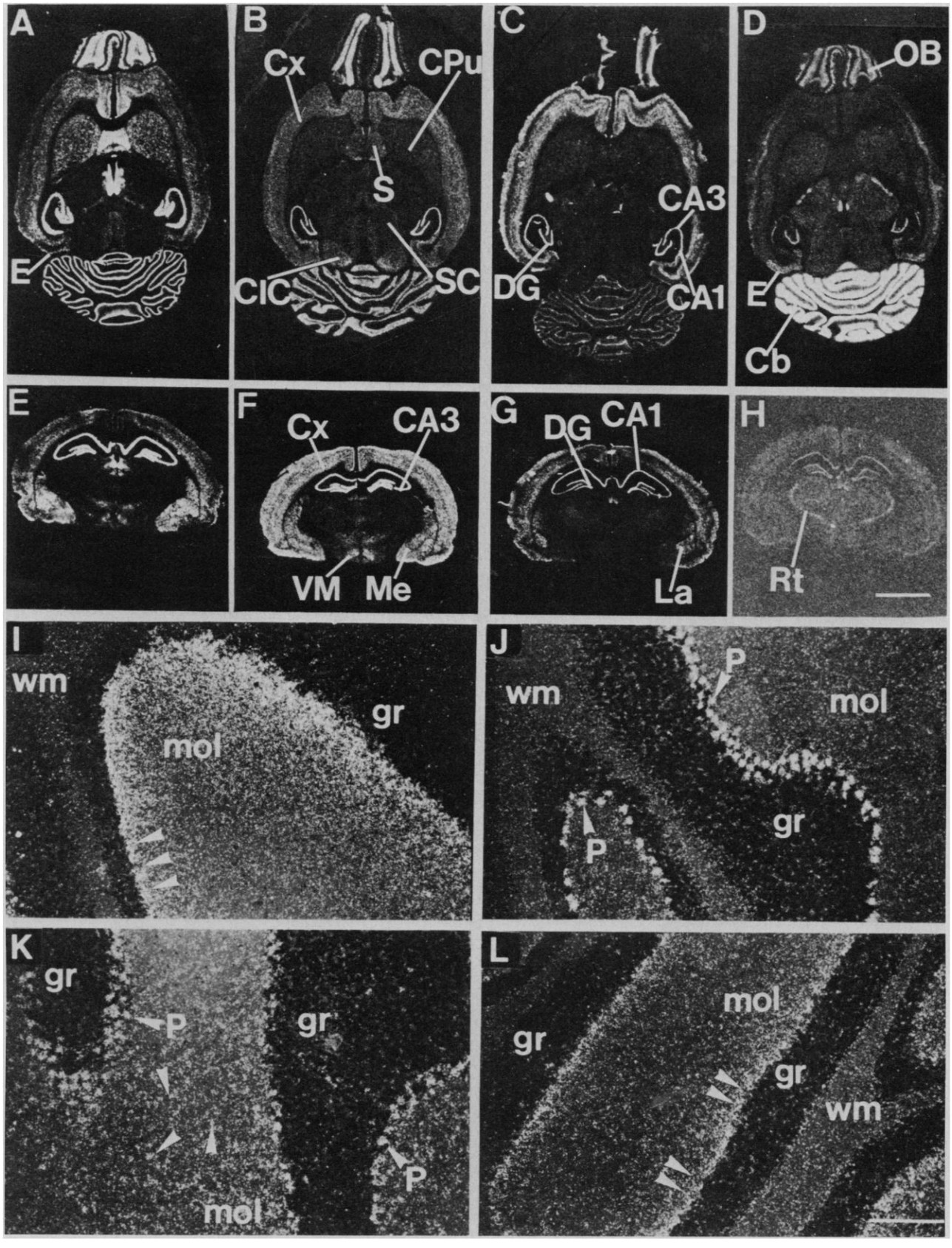
To study the pharmacology of the recombinantly expressed receptors (*10*), we performed ligand binding studies with low concentrations of [^{3}H]AMPA and [^{3}H]kainate. At 5-nM labeled ligand (*14*), only specific [^{3}H]AMPA binding was observed. Representative results obtained from GluR-B (Fig. 3) show that [^{3}H]AMPA bound to a single class of high-affinity sites having a dissociation constant (K_d) of 12 ± 2 nM (n = 3), in good agreement with the high-affinity [^{3}H]AMPA site in brain membranes (*17, 18*). Binding was most effectively competed by quisqualate, followed by glutamate, and least effectively by kainate. This order of potency for displacement is diagnostic of the AMPA receptor (*14, 17*) and was displayed by each of the four receptors and the combination of Glu-R-A and -B. This order differs from that of the high-affinity kainate receptors, which show a rank order of kainate>quisqualate>glutamate (*19*). Moreover, CNQX inhibited [^{3}H]AMPA binding at GluR-B with an inhibitory constant of 320 nM, close to the value reported for the rat cortical AMPA receptor (*14*). Hence, the pharmacological properties displayed by GluR-A, -B, -C, and -D are hallmarks of AMPA receptors.

The pattern of receptor expression in rat brain as visualized by in situ hybridization revealed that the mRNAs encoding the receptors are expressed prominently throughout the neuroaxis, with detailed differences apparent in their regional distribution (Fig. 4). In cerebral cortex, the expression patterns of GluR-A, -C and -D mRNAs clearly differ among layers, while GluR-B mRNA is uniformly found in all layers. Only low levels of GluR-A and -C mRNA occur in layers III and IV, whereas GluR-D expression appears to be elevated in this region. In the entorhinal cortex, GluR-A mRNA is conspicuously low (Fig. 4A), while GluR-D mRNA is prominent in this area (Fig. 4D). All mRNAs are found in caudate-putamen. In the hippocampus, the GluR-A, -B, and -C mRNAs are abundantly expressed in dentate gyrus and the pyramidal cell layer, with no apparent gradient of expression between CA1 and CA3. In contrast, GluR-D mRNA levels are relatively high in CA1 and in dentate gyrus but diminish significantly in CA3-CA4.

Other differences in regional expression patterns are seen in the hypothalamic-thalamic-amygdaloid areas. The GluR-A and -B mRNAs are expressed in hypothalamic nuclei such as the ventral medial area, where GluR-C and -D mRNA expression is scarce (Fig. 4, E to H). Thalamic mRNA levels of all the receptors are low, although the GluR-D gene seems to be the most ubiquitously expressed in thalamus, with particularly high levels in the reticular thalamic nucleus (Fig. 4, D and H). In the amygdala, GluR-A and -B mRNAs are abundant in all nuclei, whereas GluR-C mRNA is found in more restricted locations such as the lateral amygdaloid nucleus.

The differential cellular distribution of the four mRNAs is illustrated in cerebellum. Low power darkfield (Fig. 4, I through L) and high-power brightfield (not shown) reveal for GluR-A mRNA a continuous heavy line of silver grains, indicative of simultaneous expression in the interspersed Purkin-

Fig. 4. In situ hybridization of AMPA glutamate receptor mRNAs in horizontal and coronal sections of rat brain. (**A** and **E**) GluR-A distribution; (**B** and **F**) GluR-B (**C** and **G**) GluR-C; (**D** and **H**) GluR-D. Cb, cerebellum; CIC, central nucleus of the inferior colliculus; CPu, caudate putamen; Cx, cortex; DG, dentate gyrus; E, entorhinal cortex; La, lateral amygdaloid nucleus; Me, medial amygdaloid nucleus; OB, olfactory bulb; Rt, reticular thalamic nucleus; S, septal nuclei; SC, superior colliculus (deep layers); VM, ventral medial thalamic nucleus. Exposure time was 1 week on Kodak XAR-5 film. (H) is printed lighter to facilitate visualization of detail. (I through L) Low power, darkfield photomicrographs of cerebellum. (**I**) GluR-A distribution; arrowheads indicate continuous line of silver grains along the Purkinje-Bergmann layer. (**J**) GluR-B; arrowheads indicate labeled Purkinje cell. (**K**) GluR-C; unlabeled arrowheads indicate clusters of silver grains in molecular layer over stellate-basket cells. (**L**) GluR-D; arrowheads as in (I). gr, granule cell layer; mol, molecular layer; P, Purkinje cells; wm, white matter. In situ hybridization was performed as described (*27*). Scale bars: (H), 3.7 mm; (L), 500 μm.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je and Bergmann glia cells. From this line of cells, silver grains spread into the molecular layer, presumably tracing the extended Bergmann glia cytoplasm (*20*). The granule cells are not decorated by the GluR-A probe. The GluR-B mRNA is present at high levels in Purkinje cells and granule cells but not Bergmann glia. High-power brightfield reveals many silver grains over the granule cells, but the heavy thionin stain precludes grain reflection under darkfield (Fig. 4J). The GluR-C gene is expressed in Purkinje cells, stellate-basket cells, and possibly in Golgi type II cells, with no detectable expression in granule cells or Bergmann glia cells. GluR-D mRNA is heavily expressed in granule cells as observed under high-power brightfield, in some stellate-basket cells residing in the inner half of the molecular layer, and in putative Bergmann glia. Purkinje cells do not seem to express this mRNA.

Overall, the spatial pattern and extent of expression of the four mRNAs largely match [^{3}H]AMPA binding in telencephalic regions (*1, 17, 21*), while high affinity [^{3}H]kainate sites seem to be more restricted and are localized to hippocampal CA3 areas, deep cortical layers, striatum, and reticular thalamic nuclei (*22*). However, a few discrepancies between [^{3}H]AMPA sites and mRNA expression should be noted. The hippocampal CA3 area exhibits lower levels of [^{3}H]AMPA binding than does CA1 (*1, 17, 21*). Yet GluR-A, -B, and -C mRNAs are equally abundant in both areas. Additionally, [^{3}H]AMPA binding is not predominant in the reticular thalamic nucleus (*1, 17, 21*), although GluR-D mRNA is particularly prominent in this region. Finally, [^{3}H]AMPA binding in cerebellum is low compared to cortical-hippocampal areas, although the receptor mRNAs are prominent in cerebellum. Some of these findings could be rationalized by postulating the existence of presynaptic autoreceptors, spatially distant from the cell body. Alternatively, the polypeptides presented here might participate in the formation of pharmacologically uncharacterized receptors. Clearly, a reclassification of glutamate receptors on a molecular basis is warranted.

In conclusion, our results demonstrate the existence of multiple glutamate receptors that display characteristic AMPA pharmacology and that are abundantly and differentially expressed in the brain. By demonstrating that a receptor is responsive to both AMPA and kainate, our results lend direct support to the evolving concept that many of the electrophysiological effects of kainate are, in fact, mediated by AMPA receptors (*16, 23*). In concert with a new generation of highly selective antagonists currently under development (*2, 24*), the cloned AMPA receptor cDNAs should lead to a better understanding of the role of excitatory amino acid receptors in the normal and diseased central nervous system.

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25. Single-letter abbreviations for the amino acid residues are A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
26. K. B. Mullis and F. A. Faloona, *Methods Enzymol.* **155**, 335 (1987). The primers 5′GCGAATTC-GT(G,C) (G,A) (G,A) (G,A) GA (T,C) GG (G,A,T,C)-AA(G,A)TA(T,C)GG-3′ (upstream primer, sense), and 5′GCGGTACC(G,T,C)A(A,T)(G,A)G(A,T)-(G,A)AA(G,C)A(G,A,C)CCACCA-3′ (downstream primer, antisense), were constructed to the peptide sequences V(G,K,S)DGKYG [upstream; residues 641-647 in GluR-A (Fig. 1)] and WW(F,L,V)F-(S,T)(I,L) [downstream; within TM3 (Fig. 1)]. Reactions (10 mM tris-HCl, pH 8.7, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM each of dGTP, dATP, dCTP, dTTP) contained, in 50 μl, 20 ng of rat brain cDNA, 50 pmol of each primer and 1 U *Thermus aquaticus* DNA polymerase (Perkin-Elmer/Cetus). Forty cycles (94°C, 0.6 min; 55°C, 0.2 min; 72°C, 0.7 min) were performed, with a programmable thermocycler (Techne, Cambridge, U.K.).
27. W. S. Young, E. Mezey, R. E. Siegel, *Neurosci. Lett.* **70**, 198 (1986); W. Wisden, B. J. Morris, M. G. Darlison, S. P. Hunt, E. A. Barnard, *Neuron* **1**, 937 (1988). Briefly, 45-nucleotide antisense probes designed to a region within the sequence divergent loop between TM1 and TM2 (A, 5′-GTCACTG-GTTGTCTGGTCTCGTCCCTCTTCAAACT-CTTCGCTGTG-3′; B, 5′-TTCACTACTTTGTGT-TTCTCTTCCATCTTCAAATTCCTCAGTG-TG-3′; C, 5′-AGGGCTTTGTGGGTCACGAGGT-TCTTCATTGTTGTCTTCCAAGTG-3′; D, 5′-CT-GGTCACTGGGTCCTTCCTTCCCATCCT-CAGGTTCTTCTGTGTG-3′) were 3′-labeled by the addition of 10 to 30 [^{35}S]dAMP residues (α [^{35}S]dATP, 1200 Ci/mmol, New England Nuclear). Hybridization was in 50% formamide in 4× standard saline citrate (SSC), 10% dextran sulfate at 42°C with probe used at 1 pg/μl. Sections were washed in 1× SSC and 60°C. Parallel experiments performed with a 20-fold excess of unlabeled probe in addition to labeled probe resulted in no signal. Nonspecific labeling of tissue resulted in a uniform grey background with white and grey matter tracts being indistinguishable. Additionally, oligonucleotides constructed to different parts of the mRNAs and higher stringency conditions (0.1× SSC, 60°C) all gave results consistent with those presented here. Sections were dipped in Kodak NTB2 emulsion, stored dessicated at 4°C for 5 weeks, and developed in Kodak D19 developer. After fixation, sections were counterstained with thionin before mounting and viewing through a Zeiss Axioplan microscope. Structures were identified according to I. G. Paxinos and C. Watson [*The Rat Brain in Stereotaxic Coordinates* (Academic Press, Sydney, ed. 2, 1986)].

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