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Diversity of Glutamate Receptor Subunit mRNA Expression within Live Hippocampal CA1 Neurons

SCOTT A. MACKLER[1] and JAMES H. EBERWINE

Departments of Pharmacology (S.A.M., J.H.E.) and Psychiatry (J.H.E.), University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104–6084

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SUMMARY

Glutamate-mediated neurotransmission occurs through the activation of multimeric postsynaptic receptors. One mechanism by which functional diversity of glutamate responsiveness may occur is by a single cell expressing multiple receptors containing different subunits. In a direct test of this hypothesis, we examined the glutamate receptor subunit mRNA composition of several individual CA1 neurons in hippocampal slices. Experiments used amplified antisense RNA coupled with expression profiling and polymerase chain reaction amplification to identify and determine the relative amounts of subunit mRNAs co-localized in single cells. The results demonstrate that each CA1 neuron contains varying amounts of most glutamate receptor mRNAs. In addition to relative mRNA levels, the single-cell approach also highlighted other possible sources of receptor diversity. This included the existence of novel, alternatively spliced forms of the *N*-methyl-D-aspartate receptor type 1 and glutamate-kainate receptor type 2 subunits. Surprisingly, levels of *N*-methyl-D-aspartate receptor type 1 mRNA were relatively low, compared with those of other glutamate receptor mRNAs. One postulated source of potential heterogeneity, RNA editing, was not a general cellular mechanism. There was no evidence that glutamate receptor type 5 mRNA was edited in any of the cells that were examined. These data show that individual CA1 neurons, in the intact synaptic network of hippocampal slices, generate glutamate receptor mRNA diversity in several ways, which together contribute to the diversity of functional receptors observed electrophysiologically.

Glutamate is the principal neurotransmitter candidate at most central nervous system excitatory synapses. It initiates its physiological activity by binding to EAA receptors at the postsynaptic cell surface. Historically, glutamate receptors were classified into three categories based upon agonist affinities, i.e., NMDA, AMPA, and kainate receptors (1). Electrophysiological recordings from cells in primary cultures and live slice preparations (2–8) have documented a tremendous diversity of conductances and kinetics after glutamate receptor activation. This has led to the hypothesis that native EAA receptors exist in multiple forms (4, 9). Electrophysiological investigations of multiple receptors are limited by the lack of specific agonists and antagonists that distinguish among hetero-oligomeric receptors.

Another approach to studying single-cell receptor diversity is to use molecular biological techniques to determine which EAA receptor subunit mRNAs are simultaneously present in individual cells. Molecular cloning of the glutamate receptors indicates that there are at least 14 individual subunits (10–18). Some of these subunit proteins were demonstrated to produce *in vitro* hetero- and homomeric complexes with diverse functional and pharmacological responses to EAA agonists (10–19). Thus, the diversity of glutamate responsiveness may be due, in part, to cellular expression of multiple glutamate receptor mRNAs, giving rise to an array of distinct receptor channels. If receptor diversity is generated in the central nervous system by expression of multiple receptor subunit genes, then cellular co-localization of multiple receptor subunit mRNAs is required. Evidence supporting this hypothesis has consisted of *in situ* hybridization studies using a limited number of receptor probes in serial tissue sections (12, 16, 18, 20).

The present experiments were designed to demonstrate which glutamate receptor subunit mRNAs are simultaneously present in individual hippocampal CA1 neurons in live slice preparations and to determine their relative abundances. In addition, examination of mRNA within single neurons was

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[1] Present address: Department of Medicine, University of Pennsylvania Medical School, Philadelphia, PA 19104.

ABBREVIATIONS: EAA, excitatory amino acid; AMPA, α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid; NMDA, *N*-methyl-D-aspartate; APV, D-amino-2-phosphonovalerate; EPSC, excitatory postsynaptic current; GFAP, glial fibrillary acidic protein; SSC, standard saline citrate; aRNA, antisense RNA; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; bp, base pair(s); GluR-1–6, glutamate receptor types 1–6; NMDAR1 and -2, *N*-methyl-D-aspartate receptor types 1 and 2; KA2 and -2, kainate receptor types 1 and 2.

used to determine whether post-transcriptional editing of glutamate receptor mRNA (21, 22) occurs. The present results provide insight into the degree of receptor subunit mRNA diversity present within individual hippocampal neurons.

Materials and Methods

Slice preparation and cDNA synthesis. Sprague-Dawley rat pups (10–20 days of age) were used in this study. Animals were sacrificed by decapitation and 200-μm-thick coronal sections of the hippocampus were removed and studied as described previously (23, 24). A whole-cell configuration was formed by aspiration and the cells were maintained under voltage-clamp conditions at a resting potential of −70 mV. Because of the complexity of pharmacological responsiveness of the glutamate receptor system, the functional nature of the expressed channels in the examined pyramidal cells was not determined. We have determined that at least some of the cells express functional NMDA receptors, because of the ability of APV to block use-dependent enhancement of EPSCs (24). Pharmacological characterization of glutamate receptors in live slice preparations is also hampered by the difficulty of eliciting selective cellular responses, due to the lack of selective ligands and drug diffusion problems. Furthermore, primary cellular effects are difficult to separate from secondary cell influences. Synthesis of cDNA from cellular mRNA was initiated by bringing the reagents that are necessary for cDNA synthesis into contact with the cellular mRNA *in situ*. This was accomplished by application of these components via passive diffusion from a preloaded patch pipette. After varying time periods (5–15 min) the cellular contents were removed by aspiration, a process that was monitored visually. Completion of cDNA synthesis proceeded *in vitro* at 37° for 60–90 min. Each of the single-stranded cDNAs contained a T7 RNA polymerase promoter sequence. The cDNA molecules were next converted into a double-stranded template, with removal of unincorporated deoxynucleotide triphosphates (25). The double-stranded cDNA was processed into amplifiable template as described (25).

aRNA amplification and expression profiles. Approximately 20% of the solution of cDNAs from each cell underwent *in vitro* transcription (24, 25). The aRNA was isolated by phenol-chloroform extraction and ethanol precipitation, heat-denatured (95° for 5 min), and hybridized to Southern blots at 42° for 48 hr. The Southern blots contained equimolar amounts of linearized cDNAs for the glutamate receptors, low molecular weight neurofilament, and GFAP. The hybridization mixture contained 5× SSC, 5× Denhardt's solution, 100 μg/ml sheared DNA, and 50% formamide. Blots were washed at a final concentration of 0.2× SSC/1% SDS at 42°, air-dried, and apposed to autoradiographic film. The autoradiograms were analyzed using a scanning laser densitometer and the results were calculated as described previously (24).

The level of any particular mRNA was not quantified in absolute terms, but the relative ratio of the abundance of multiple mRNAs to neurofilament mRNA abundance was determined. This normalization permits analysis of selected mRNA levels in comparison with multiple other mRNAs from the same sample (24, 26). The population of aRNAs were processed so that the composite aRNAs were handled similarly. Variables due to differences in efficiencies of enzymatic reaction, etc., were minimized by this procedure.

Efficiency of *in vitro* amplification of glutamate receptor cRNAs. In separate experiments, 5-fold and 10-fold greater amounts of sense cRNAs from linearized cDNAs encoding the glutamate receptor subunits GluR-1–6 underwent the same enzymatic reactions as did material from the single cells, to measure the efficiency of amplification. Ten to 100 ng of full length cRNAs were transcribed into first-strand cDNAs using the T7 polymerase promoter-oligothymidine primer. These cDNAs were made into double-stranded templates and equivalent volumes were amplified by *in vitro* transcription (24, 25). The reactions, phenol-chloroform extractions, and ethanol precipitations for each cRNA were performed in separate tubes.

Conditions of PCR for individual subunit mRNAs. Single-stranded cDNAs from individual cells were prepared as described above. Heat-denaturated, single-stranded cDNA was added to a mixture containing 10 mM Tris, pH 8.3, 50 mM KCl, 2 mM $MgCl_2$, 250 μM levels of dATP, dGTP, dCTP, and TTP, 5 units of AmpliTaq DNA polymerase, and 500 ng of each of the two primers. Conditions for PCR were selective for different sets of primers and consisted of 30–35 cycles of 1) denaturation (95° for 1 min), 2) primer annealing (ranging from 45° to 54° for 1–2.5 min), and 3) extension (72° for 1.5–2 min), followed by a final extension at 72° for 3 min. The primers ranged from 20 to 27 bases in length and were chosen from the following nucleotide sequences (oligonucleotide name/nucleotide at the 3′ or 5′ end of the oligonucleotide): GluR-2, 2–1/2065, 2–2/2448, 2–3/2545, 2–4/2608 (FLOP), 2–5/2856, 2–6/3145, 2–7/2608 (FLOP), 2–8/3064, and 2–9/3245; GluR-4, 4–1/2390 and 4–2/2842; GluR-5, 5–1/1708 and 5–2/2280; GluR-6, 6–1/1792, 6–2/2355, 6–3/2355, and 6–4/2720; KA2, K-1/predicted amino acid 743 and K-2/predicted amino acid 904; NMDAR1, NM1–1/2472 and NM1–2/2739; and NMDAR2 (numbered from NR2A but will also hybridize to NR2B), NM2–1/predicted amino acid 799 and NM2–2/predicted amino acid 943.

Knowledge of the intron/exon splice sites was used to design oligonucleotides for PCR amplification of GluR-5 and NMDAR1. This information was not known for the KA2 receptor; hence, it was impossible to predict whether alternative splicing may occur. The PCR-amplified products are, however, unlikely to result from genomic DNA amplification, because there is no genomic DNA present in samples resulting from the use of this technique, as determined by the absence of middle repetitive elements in the aRNA population.

Confirmation of the PCR products. PCR products from individual reactions were size-separated in agarose gels, denatured with 0.2 N NaOH, neutralized with 0.2 M Tris, pH 7.0, and transferred to nitrocellulose filters by capillary diffusion. This was followed by hybridization of either full length cDNA clones (random primed with [α-^{32}P] dCTP to a specific activity of $>10^8$ cpm/μg; GluR-2, GluR-4, GluR-6, and NMDAR1) or oligonucleotides located between the primer pairs used for PCR (end-labeled with [γ-^{32}P]ATP to a specific activity of $>10^9$ cpm/μg; KA2). Probes were hybridized for 24–72 hr at 68° (cDNAs) or 54° (oligonucleotides). The Southern blots were washed to a final stringency condition of 0.1× SSC/1% SDS at 60° (for full length cDNA probes) or 0.2× SSC/1% SDS at 42° (for internally directed oligonucleotides). The blots were air-dried and apposed to autoradiographic film for 4–72 hr. Also, the DNA sequences of selected PCR products were determined to further ensure PCR band specificity and to test for the occurrence of RNA editing.

Results

Expression profiles of mRNA encoding the glutamate receptor subunits GluR-1 through GluR-6. The experimental outline is depicted in Fig. 1. A recording microelectrode containing the reagents necessary for cDNA synthesis was used to perform whole-cell recording on individual CA1 neurons in live slice preparations (23, 24). The CA1 neurons (identifiable by their location and morphological appearence) exhibited EPSCs upon stimulation of the stratum radiatum (an illustrative example is shown in Fig. 1). The EPSCs persisted in the presence of a NMDA-specific channel blocker (APV) (1), showing that non-NMDA receptors contributed to the synaptic responses. The ability to stimulate EPSCs ensured that the CA1 neurons remained viable during the period of study and that at least one type of presynatic input was present in the slice preparation. cDNA was made from the cellular mRNA using an oligo(dT) primer that was extended at the 5′ end with a T7 RNA polymerase promoter site. The population of cDNAs was made double-stranded and was then copied into amplified aRNA using T7 RNA polymerase (24, 25). The radiolabeled

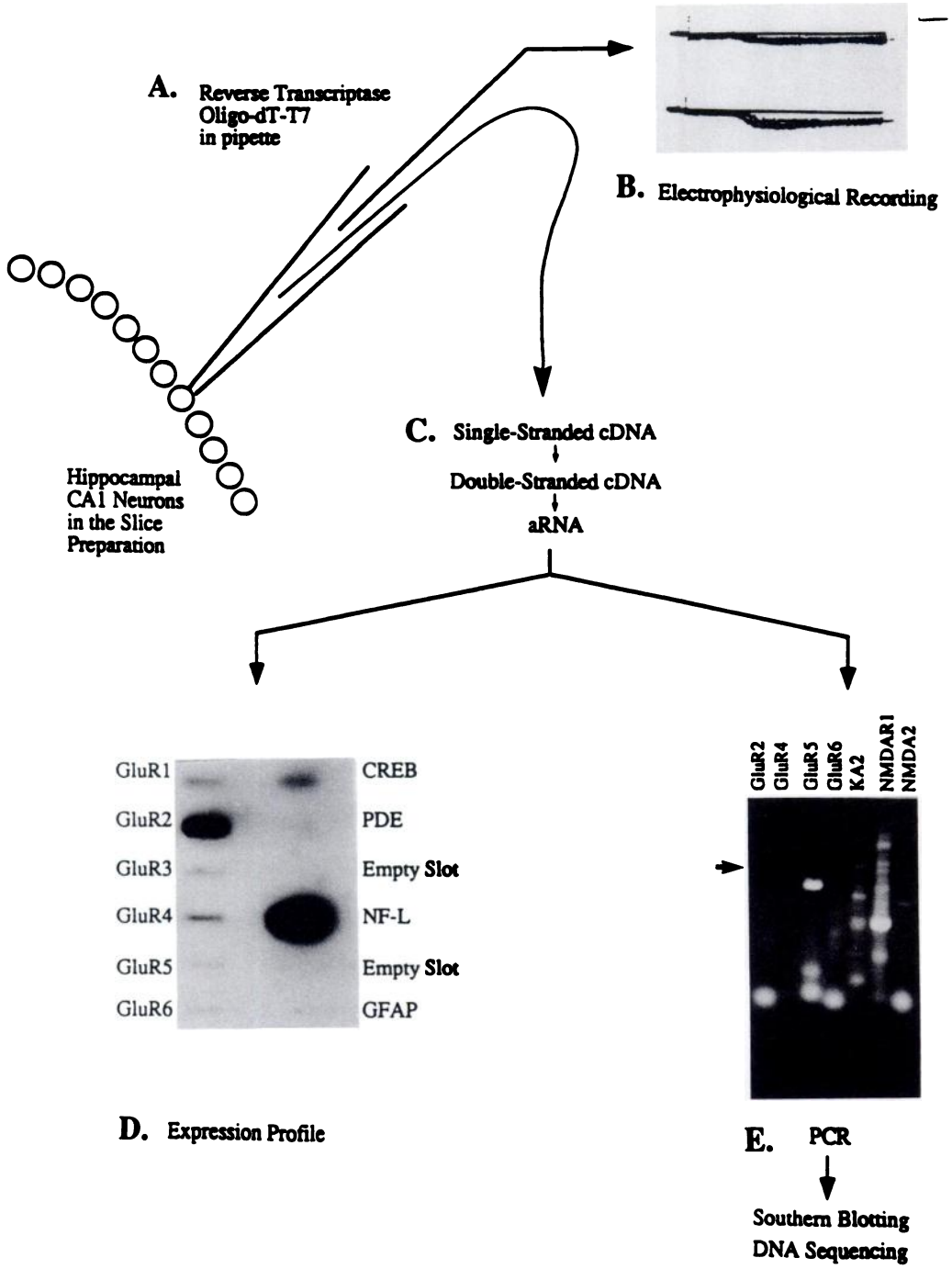

Fig. 1. Experimental design. A, Coronal sections of the hippocampus (100–200-μm thick) were prepared for electrophysiological study. Patch-clamp recordings using the whole-cell configuration used microelectrodes containing the reagents necessary for first-strand cDNA synthesis (23, 24). B, CA1 neurons, identified by their morphology, position, and response to extracelluar stimulation of presynaptic Schaffer fibers, were maintained under voltage-clamp at −70 mV for >5 min. *Upper trace*, EPSC evoked in the presence of 100 μM APV. The EPSC must therefore result from the activation of non-NMDA glutamate receptors. *Lower trace*, evoked EPSC from another cell in the same slice without APV in the perfusate. *Calibration bars*, 0.25 nA and 1 msec. These data are included to show that whole-cell recordings can be performed from the same cells in which the poly(A)$^+$ RNA is amplified. C, The oligonucleotide used for priming cDNA synthesis consisted of an oligo(dT) sequence with the bacteriophage T7 RNA polymerase promoter sequence located at the 5′ end (25). This oligonucleotide allows for the linear amplification of the poly(A)$^+$ RNA population of an individual cell. D, The amplified aRNA may be used directly as a probe of multiple cDNAs immobilized on nitrocellulose filters. This results in a profile of the relative amounts of the expressed RNAs encoded by these cDNAs. In this representative expression profile, the *left column* contained (from *top* to *bottom*) cDNAs encoding GluR-1, GluR-2, GluR-3, GluR-4, GluR-5, GluR-6, and NMDAR1 and the *right column* contained the cAMP response element-binding protein (*CREB*), the rat homologue for a *Drosophila* phosphodiesterase (*PDE*), an empty slot, low molecular weight neurofilament (*NF-L*), an empty slot, and GFAP. The cDNAs were linearized with *Bam*HI and applied to a slot-blot apparatus in equimolar amounts. The differences in intensities of the hybridization signals reflect the relative abundances of each mRNA from the same neuron. There is little cross-hybridization of the cDNA clones encoding the glutamate receptors with one another. E, Single-stranded cDNA synthesized from aRNA of individual cells was used as a template for PCR amplification of specific glutamate receptor subunits. It should be emphasized that the PCR as used in this study is not quantitative. The PCR band intensity is therefore not proportional to the abundance of the corresponding mRNAs. Each glutamate receptor subunit tested for was present in the majority of the examined CA1 neurons. In addition, multiple PCR products were apparent after use of several specific primer pairs (NMDAR1 and KA2). In lanes where there are multiple bands some are novel forms of the amplified subunits (see Fig. 3). *Arrow*, position where a band of 600 bp would migrate in this 2.5% agarose gel. Twenty-five CA1 neurons from nine Sprague-Dawley rat pups (age, 10–20 days) were used in this study.

aRNA population was used as a hybridization probe for several glutamate receptor cDNAs, to obtain an expression profile of relative abundances (Fig. 1D) (24–27). As a specificity control, the aRNA did not hybridize to a cDNA for GFAP (a specific glial cell marker) (28) that was included in the expression profile, indicating that the amplified mRNA was derived from neurons. Additionally, this control showed that there was no glial contamination of the microelectrode during penetration of the tissue slice; otherwise, GFAP should have been amplified. The formation of a gigaseal represents a mechanical barrier that prevents any mRNAs in the surrounding tissue from entering the microelectrode that is used for cDNA synthesis. Other controls for the range of sensitivity of aRNA amplification included cDNAs encoding cAMP response element-binding protein and a rat phosphodiesterase (PDE), both of which are present in CA1 neurons at differing levels. The most intense hybridization signals were present in the slots containing GluR-2 and GluR-4, two AMPA-selective receptors (Fig. 1D). Quantification of expression profiles obtained from 21 CA1 neurons by two-dimensional scanning densitometry revealed that multiple glutamate receptor mRNAs simultaneously exist in differing amounts within individual cells (Table 1). In Table 1, data generated for two single cells are compared with the aggregate of results from 21 individual CA1 pyramidal cells. *In vitro* transcription using 5–10-fold greater amounts of cRNAs encoding GluR-1–6 demonstrated the linearity of efficiency of amplification. The absolute values, based upon the incorporation of [α-^{32}P]CTP, were used in mathematical corrections for the relative values of each receptor subunit. These corrections were 3.33 (GluR-1), 1.74 (GluR-2), 1.35 (GluR-3), 0.79 (GluR-4), 1.36 (GluR-5), and 1.06 (GluR-6).

The mRNA for NMDAR1 (15) is present in very low levels, compared with the non-NMDA glutamate receptors (data not shown), as determined by similar expression profiles. Confirmation of the presence of NMDAR1 by another amplification strategy (see below) indicates that this receptor subunit is present in CA1 neurons but in very low abundances, compared with the non-NMDA glutamate receptor subunit mRNAs.

Characterization of isoforms of the multiple glutamate receptor subunit mRNAs. Amplification of aRNA is useful for measuring the relative amounts of several mRNAs. However, characterization of multiple forms of receptor subunits that share significant sequence similarity would be difficult to perform using aRNA and expression profiling alone. If appropriately specific cDNA clones have not been generated, then cross-hybridization between multiple forms of the receptors may occur. This problem can be avoided by adding additional specificity to the amplification process. This is accomplished by using the cDNA made from single-cell-derived aRNA as a template for amplification using sequence-specific primers in the PCR (29, 30). Therefore, to determine whether alternative forms of these receptors exist within a single cell, the population of aRNAs from individual CA1 neurons was first converted into a single-stranded cDNA population (Fig. 1C). This cDNA was then used as a template for PCR to amplify specific receptor sequences. It should be emphasized that the PCR, as used in this study, was not quantitative. The PCR band intensity is therefore not indicative of the abundance of the corresponding mRNA. Sense and antisense oligonucleotides were used as primers for amplification of portions of GluR-2 (140 bp), GluR-4 (460 bp), GluR-5 (585 bp), GluR-6 (385 bp), KA2 (a kainate-selective glutamate receptor subunit) (483 bp) (16), NMDAR1 (307 bp), and NMDAR2A and -2B (432 bp) (Fig. 2) (18). PCR was successful for each of these receptors in the majority of the cells examined (Fig. 1E; Table 2). Direct DNA sequencing of subclones of the PCR products, as well as hybridization of internally directed oligonuceotide or full length cDNA probes to Southern blots containing the PCR products (Fig. 3), demonstrated that the amplification was specific for GluR-2, GluR-4, GluR-5, GluR-6, KA2, and NMDAR1. PCR with NMDAR2 primers produced an amplified product of the predicted size, suggesting the presence of cellular mRNAs for these receptors.

TABLE 1

Relative amounts of glutamate receptors in CA1 neurons

The expression profiles from a total of 21 single cells were analyzed with a scanning laser densitometer. The value for each receptor was calculated as a percentage of that obtained for low molecular weight neurofilament (NF-L), which was chosen as an internal reference value (24). Multiple exposure times were used to avoid saturation of the low molecular weight neurofilament signal. For the population of cells, GluR-2 and GluR-4 were present in the largest relative amounts. Autoradiographic signals for NMDAR1 were <1% of the low molecular weight neurofilament signal in seven of the cells and were absent in the remainder, indicating the low abundance of this mRNA. The individual percentages for two single cells, HP42 and HP38, are shown as examples of the type of single-cell data that are generated. In parentheses are relative percentages calculated by using the efficiency of amplification of individual glutamate receptor subunit cRNAs. Five-fold and 10-fold greater amounts of GluR-1-6 cRNAs were processed independently in a manner identical to that used for the population of poly(A)$^+$ RNAs in single cells.

Glutamate receptor	Relative amount		
	HP42	HP38	Total of 21 cells
	% of NF-L signal		
GluR-1	1.1 (3.67)	2.5 (8.33)	1.46 ± 0.50 (4.86)
GluR-2	33 (57.42)	53.6 (93.26)	23.80 ± 8.31 (41.40)
GluR-3	1.4 (1.89)	1.7 (2.30)	2.70 ± 1.04 (3.65)
GluR-4	6.5 (5.14)	7.6 (6.00)	8.96 ± 1.44 (7.08)
GluR-5	0.9 (1.22)	1.5 (2.04)	4.08 ± 1.48 (5.55)
GluR-6	0.6 (0.64)	0.9 (0.95)	5.49 ± 2.09 (5.82)

Discussion

Several cDNAs have been cloned that encode EAA receptor subunits (10–18) in neurons. Many of the receptor subunit proteins encoded by these mRNAs are able to interact to form functional hetero-oligomers *in vitro* (3, 5, 6, 8, 9). Electrophys-

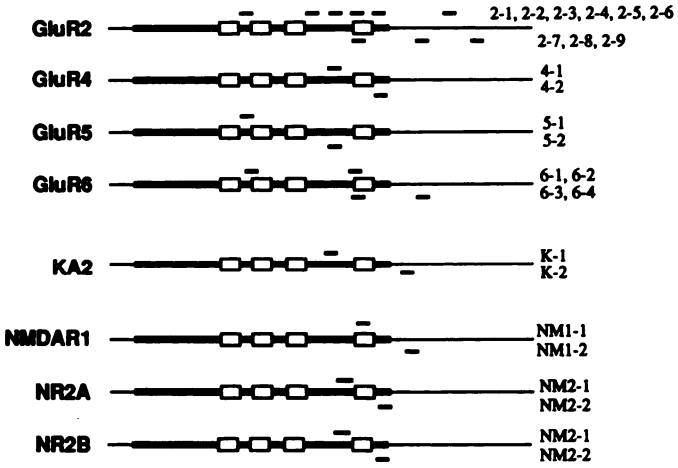

Fig. 2. Strategies for PCR amplification. The location and orientation of the oligonucleotides included in the PCR are depicted as *bars above* (sense) and *below* (antisense) the respective cDNA clones. The distances between primer pairs ranged from 86 to 1180 bases. *Thick lines*, translated portions of the mRNA; *thin lines*, untranslated regions; *open boxes*, putative membrane-spanning regions. This diagram is not drawn to scale.

TABLE 2

Detection of receptor subunit mRNAs in CA1 neurons

The results obtained from 21 CA1 neurons, using a combination of expression profiles and PCR amplification, are contained here. AMPA and kainate receptors are classified on the basis of highest agonist affinity, as determined in other studies. NMDAR2 data may contain contributions from amplified products for NMDAR2A or -B. These classifications do not distinguish between already published sequences and novel, alternatively spliced products (discussed in the text). Data from expression profiles are located to the left of the slash and data from PCR experiments are located to the right of the slash. In the majority of the cells examined, many of the AMPA, kainate, and NMDA glutamate receptor subunit mRNAs were co-localized. cDNA clones for the KA2 and NMDAR2 subunits were not available for use in the expression profiles (results in these columns are from PCR amplification alone). +, Confirmation of the presence of this subunit mRNA in a CA1 cell; n, inability to detect this subunit mRNA (PCR was performed a minimum of two times for the majority of the listed results). See text for discussion.

	AMPA		Kainate			NMDA	
	GluR-2	GluR-4	GluR-5	GluR-6	KA2	NMDAR1	NMDAR2
HP10	+/+	+/n	+/+	+/n	+	+/+	n
HP19	/+	/+	/+		n	/+	+
HP24	/+		/+		+	/+	
HP25	+/n	n/	+/+	+/n	+	+/+	+
HP29	+/n	+/+	n/+	n/	n	n/n	+
HP30	/+		/+		n	/+	+
HP31	/+	/+	/+		n	/+	+
HP32	+/	+/	+/+	+/+	n	+/+	n
HP33	+/	+/	n/+	+/+	+	n/+	n
HP34	+/+	+/n	+/+	+/n	+	n/+	n
HP35	+/+	+/n	+/+	+/n	n	n/+	n
HP38	+/+	+/n	+/+	+/+	+	n/+	n
HP39	+/+	+/n	+/	+/n	+	n/+	n
HP40	+/+	+/n	+/+	+/+	n	n/+	n
HP41	+/	n/	n/+	n/	+	n/+	
HP43	+/+	+/+	n/+	n/+	+	n/+	n
HP44	+/n	+/+	+/+	+/+	+	+/	n
HP45	+/n	+/+	+/+	+/n	+	+/+	+
HP46	+/+	+/+	+/+	+/+	+	+/+	+
HP47	+/+	+/	+/+	+/+	+	+/+	+
HP48	+/+	+/	n/+	n/+	+	n/+	+

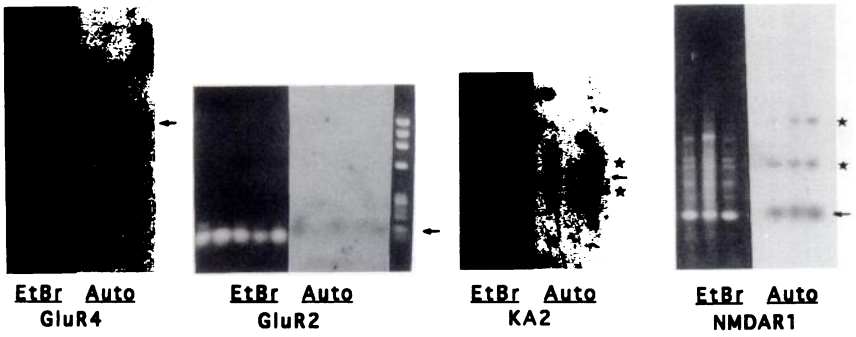

Fig. 3. Specificity of the PCR products. PCR products from single reactions were separated by agarose gel electrophoresis, followed by visualization of amplified DNAs by ethidium bromide staining (*EtBr*). *Arrows*, PCR products of predicted size that hybridized to the probe; ★, additional PCR products that hybridized to the probes (*Auto*). DNA from ϕX 174 digested with *Hae*III is included as a size marker next to GluR-2. The blots were washed at a high stringency, air-dried, and apposed to XAR film at −70° for 12 hr, with an intensifying screen. For GluR-4 results from two cells are shown, for GluR-2 results from five cells, for NMDAR1 results from three cells, and for KA2 results from three cells.

iological recordings of hippocampal CA1 neurons have demonstrated that these cells exhibit complex responses to glutamate stimulation. The present study, by combining expression profiles and PCR amplification with aRNA obtained from single live CA1 cells, shows that multiple glutamate receptor subunit mRNAs are simultaneously present in CA1 neurons. Receptor subunit mRNAs for GluR-2, GluR-4, GluR-5, and GluR-6 were present in >90% of single CA1 neurons (Table 2). Thus, the proteins encoded by these mRNAs may interact to form multimeric receptors with different functional properties (e.g., Refs. 12, 13, and 16). A critical determinant of the glutamate responsiveness of each single cell may be the types of multimeric receptors formed by interaction of the translated proteins encoded by the several co-localized subunit mRNAs.

Putative AMPA glutamate receptor subunit mRNAs. AMPA binds with high affinity to receptor complexes formed from GluR-1–4 proteins, and hence GluR-1–4 have been proposed to be AMPA receptors (19). The results presented in Table 1 show that all four of these subunit mRNAs are present in the examined cells. The relative levels vary from cell to cell, with the rank order being constant. This suggests that the invariant rank order of mRNA expression may be important to the glutamate responsiveness of the cells. Although the linearity of the *in vitro* amplification of individual GluR-1–6 cRNAs demonstrated small variations, the rank order of relative values for GluR-1–6 RNAs in single hippocampal neurons did not significantly change after correction (Table 1). Interestingly, the cRNA for GluR-1 did not show the same efficiency of transcription when taken through the aRNA procedure. This may be due to sequence-specific secondary structure limiting the cDNA or aRNA synthesis. This highlights the need to determine the efficiency of aRNA production when measuring basal levels of mRNA abundance. Unpublished results with dispersed hippocampal pyramidal cells[2] show that the relative abundances of the glutamate receptor subunits differ from the present results with slice preparations. This indicates that the relative abundances are biologically determined, as opposed to resulting from technical constraints.

[2] Y. Cao, M. Dichter, and J. Eberwine, unpublished observations.

Specifically, GluR-2 is the most abundant glutamate receptor mRNA present in CA1 neurons, of the receptors included in the expression profiles (Table 1), and was detected in all of the cells examined (Table 2). However, a PCR product was detected only using the most 3′ pair of oligonucleotides chosen for study (Fig. 2; primers 2–6 and 2–9). Several other combinations of primers were used (Fig. 2) but did not amplify this receptor subunit to ethidium bromide-detectable levels in any of the cells examined. This indicates that the majority of the aRNA representing GluR-2 is relatively short, compared with the total aRNA population. Because aRNA is directly transcribed from the cDNA, this result suggests that the initial cDNA corresponding to cellular GluR-2 mRNA is also relatively short. The length of the cDNA transcribed from the cellular mRNA is dependent upon the ability of the reverse transcriptase to copy the mRNA. If a strong secondary structure or a blocker of reverse transcriptase movement along the mRNA (e.g., ribosome assemblies) is present, it may interfere with longer cDNA synthesis.

The presence of GluR-1–4 in individual Purkinje cells has recently been described in culture (31). The authors used a modification of our sampling technique, relying solely upon PCR, in an attempt to quantitate the abundance of the receptors. PCR quantitation requires the inclusion of internal standards, which may be amplifed at a different rate from the unknowns, thus making quantitative measurements problematic. The difficulty of quantitating PCR and the inability to state that a particular mRNA is absent solely because it cannot be PCR-amplified using a specific set of conditions suggest that the authors examined only a fraction of the actual mRNAs present within the Purkinje cells.

Putative kainate glutamate receptor subunit mRNAs. In contrast to the GluR-1–4 subunits, to which AMPA binds with high affinity, kainate binds with high affinity to receptors containing the GluR-5 and -6 subunits (11, 14). cDNA clones encoding other proteins that exhibit kainate binding have recently been isolated. One of these is KA2, the cDNA (16) which encodes a protein with a primary structure more similar to that of GluR-5 and GluR-6 than to those of other glutamate receptor cDNAs. Interestingly, coexpression of KA2 mRNA with GluR-5 or GluR-6 results in receptors with different pharmacological profiles (16). The KA2 receptor subunit was detected by PCR amplification in 66% of the CA1 neurons (Table 2). The use of degenerate primers from the predicted amino acid sequence of the KA2 receptor subunit revealed multiple bands after PCR (Figs. 1 and 2), which hybridized to an internally directed oligonucleotide probe (Fig. 3).

For a majority of individual CA1 cells examined in the present study, mRNA encoding GluR-5 was more abundant that mRNA encoding GluR-6. There were, however, a few cells in which GluR-6 mRNA was more abundant than GluR-5 mRNA. These data highlight the potential predictive value of knowledge of relative mRNA abundances. For example, if there is more functional GluR-5 subunit than GluR-6 subunit mRNA present in a cell that also contains KA2 subunit mRNA, then that cell may have a different kainate responsiveness than another cell with more GluR-6 than GluR-5 subunit mRNA. It is also possible that the presence or absence of KA2 mRNA may dictate the functional characteristics of kainate binding of CA1 pyramidal cells, by interactions with GluR-5 and/or GluR-6 protein. These predictions, based upon calculations of relative mRNA abundances, have yet to be tested.

GluR-5 mRNA was detected in all of the pyramidal cells that were examined (Table 2). A novel method to produce receptor heterogeneity, called RNA editing, has been postulated to occur for GluR-2, -5, and -6. A single nucleotide change resulting from RNA editing is suggested to selectively alter Ca^{2+} conductance through GluR-5 protein by switching a glutamine codon to an arginine codon in a putative transmembrane segment (21). After cloning of the 585-bp product into the PT7-Blue cloning vector (Novagen), DNA sequencing across the second transmembrane region of 18 independent clones from six different cells (seven isolates from a single cell, with four, four, one, one, and one isolates coming from other cells) revealed the presence of only a glutamine codon in the area proposed to undergo RNA editing. This result differs from a previous study in which the abundance of glutamine/arginine-containing GluR-5 was shown to be nearly 60/40 (21). A potential explanation for this difference is that the previous evidence resulted from PCR amplification of mRNA isolated from a population of heterogeneous cells rather than a single cell type as used in the present study. The results from single CA1 neurons suggest that the presumed RNA editing of GluR-5 occurs in a cell-specific manner. Sequencing of additional GluR-5 PCR products as well as GluR-2 and GluR-6, which would be expected to be edited in a manner similar to that of GluR-5 in cells where they coexist and where editing can be shown to occur, from several additional cell types will help to address this question. It also remains a formal possibility, although unlikely, that the genomic sequence at this nucleotide position differs in a cell-specific manner.

The possibility that the GluR-5 PCR product is an artifact due to amplification of genomic DNA can be discounted because the PCR primers that were made for GluR-5 were designed to span an exon-intron splice site. Because the size and sequence of the PCR product correspond to those predicted for the mature mRNA, it is clear that genomic DNA was not artifactually amplified in this procedure (30).

NMDA receptor subunit mRNAs. NMDAR1 mRNA detection via expression profiling was difficult because of its low abundance, relative to other glutamate receptor mRNAs, in CA1 neurons. However, PCR products for NMDAR1 obtained using aRNA derived from the same neurons were observed (Figs. 1 and 3). The results from both techniques indicate that the NMDAR1 subunit mRNA was present in 20 of 21 cells examined. Several PCR products were evident in the ethidium bromide-stained gel, although only three of these bands hybridized (one at the predicted size of 307 bp and two at 700 and 900 bp) to the full length NMDAR1 cDNA clone (Fig. 3). The hybridization results indicate that these three bands correspond to cDNAs of high sequence similarity with NMDAR1. The two additional hybridizing bands, shown in Fig. 3, result likely from alternatively spliced mRNAs or possibly from expression of other novel members of the NMDAR1 gene family. The existence of multiple PCR products for NMDAR1 again emphasizes the large number of functionally diverse, hetero-oligomeric receptors that may form in individual hippocampal neurons.

The CA1 neurons used in these studies exhibit synaptic potentiation (24), a use-dependent phenomenon that requires activation of the NMDA receptor. The presence of relatively low levels of NMDAR1 mRNA in CA1 cells indicates that the

amount of NMDAR1 protein necessary for potentiation does not require relatively high levels of NMDAR1 mRNA. These data highlight the caution that is necessary in making one to one correlations between mRNA and functional protein levels. The functional significance of multiple mRNA forms of the NMDAR1 subunit (as well as GluR-6 and KA2) is unclear. These novel forms may contribute to the functional heterogeneity of these receptors by altering the translational efficiency of mRNAs or by encoding proteins with different primary sequences, yielding different glutamate responsiveness. This question will be answered when full length cDNA clones for these multiple forms have been characterized and their electrophysiological responsiveness determined. Furthermore, mRNA encoding another type of NMDA receptor protein, NMDAR2, is also present in these cells (Fig. 1). The NMDAR2 PCR product is detected as a single band of the predicted size, 432 bp. The oligonucleotide primers for the NMDAR2 PCR were designed to amplify the same size band for two of the subtypes of NMDAR2 (subtypes A and B); hence, this band may represent either of these NMDAR2 forms (18).

Importantly, the results discussed above demonstrate the simultaneous presence of several types of AMPA, kainate, and NMDA receptor subunit mRNAs in single cells. It should be emphasized that mRNA levels have been examined in this paper, not functional protein levels. There are several steps separating mRNA from functional protein, including translational control and post-translational processing events. It is difficult to distinguish, at the protein level, between (for example) the functional contributions of GluR-3 and GluR-4 in heteromeric receptors in the slice preparation by using electrophysiological or pharmacological measures. Nonetheless, if relative mRNA abundances are translated into functional protein in the same relative proportions, then these results suggest that the complex electrophysiological responsiveness of hippocampal CA1 neurons to the release of glutamate by presynaptic terminals (19) may be due in part to different amounts of receptor subunit proteins.

The use of two techniques (aRNA amplification and PCR amplification) helps to ensure that mRNA encoding specific receptor subunits in single cells, when present, will be detected. In the majority of the cells having identified receptor subunit mRNAs present, the results from aRNA and PCR amplification were identical (Table 2). In some examples (e.g., results for NMDAR1), only PCR amplification was able to consistently detect the presence of the mRNAs. One explanation for this type of discordant result would be the low abundance of receptor subunit mRNAs in the population of mRNAs that were amplified from the single cell. In other examples, expression profiles revealed the presence of subunit mRNAs, whereas the PCR amplifications could not identify these mRNAs. This type of result (e.g., six of 11 cells tested with both expression profiles and PCR amplification for GluR-4) (Table 2) highlights the potential inconsistency of PCR as a quantitative technique when the material being aplified may not be full-length cDNA. This also emphasizes the fact that a negative result using PCR amplification alone does not necessarily indicate the absence of those mRNAs.

Neurotransmitter receptors assembled *in vitro* from different subunits demonstrate a wide array of responsiveness. The existence of multiple subunit mRNAs within individual neurons suggests that this same wide array of responsiveness exists *in vivo*. In particular, if the relative abundances of the different subunit proteins parallel those of the corresponding mRNAs, then knowledge of relative mRNA abundances would suggest potential stoichiometries for the assembled functional receptors. Such predictions are testable *in vivo* and *in vitro*.

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References

1. Monaghan, D., R. Bridges, and C. Cotman. The excitatory amino acid receptors: their classes, pharmacology, and distinct properties in the function of the CNS. *Annu. Rev. Pharmacol. Toxicol.* **29**:365–402 (1989).
2. Cull-Candy, S., and M. Usowicz. Multiple conductance channels activated by excitatory amino acids in cerebellar neurons. *Nature (Lond.)* **325**:525–528 (1987).
3. Jahr, C., and C. Stevens. Glutamate activates multiple single channel conductances in hippocampal neurons. *Nature (Lond.)* **325**:522–525 (1987).
4. Ascher, P., and L. Nowak. Quisqualate- and kainate-activated channels in mouse central neurons in culture. *J. Physiol. (Lond.)* **399**:227–245 (1988).
5. Llano, I., A. Marty, J. W. Johnson, P. Ascher, and B. H. Gahwiler. Patch-clamp recording of amino acid-activated responses in "organotypic" slice cultures. *Proc. Natl. Acad. Sci. USA* **85**:3221–3225 (1988).
6. Cull-Candy, S., and M. Usowicz. Whole-cell current noise produced by excitatory and inhibitory amino acids in large cerebellar neurones of the rat. *J. Physiol. (Lond.)* **415**:533–553 (1989).
7. Huettner, J. Glutamate receptor channels in rat DRG neurons: activation by kainate and quisqualate and blockade of desensitization by Con A. *Neuron* **5**:255–266 (1990).
8. Jonas, P., and B. Sakmann. Properties of AMPA subtype glutamate receptors in pyramidal cells of rat hippopcampal slices. *J. Physiol. (Lond.)* **438**:321P (1991).
9. Nowak, L. M., and P. Ascher. Electropharmacology of excitatory amino acids: counting receptors and channels, in *Allosteric Modulation of Amino Acid Receptors: Therapeutic Implications* (E. Barnard and E. Costa, eds.). Raven Press, New York, 225–232 (1989).
10. Hollmann, M., A. O'Shea-Greenfield, S. Rogers, and S. Heinemann. Cloning by functional expression of a member of the glutamate receptor family. *Nature (Lond.)* **342**:643–648 (1989).
11. Bettler, B., *et al.* Cloning of a novel glutamate receptor subunit, GluR-5: expression in the nervous system during development. *Neuron* **5**:583–595 (1990).
12. Boulter, J., *et al.* Molecular cloning and functional expression of glutamate receptor subunit genes. *Science (Washington D. C.)* **249**:1033–1037 (1990).
13. Keinanen, K., *et al.* A family of AMPA-selective glutamate receptors. *Science (Washington D. C.)* **249**:556–560 (1990).
14. Egebjerg, J., B. Bettler, I. Hermans-Borgmeyer, and S. Heinemann. Cloning of a cDNA for a glutamate receptor subunit activated by kainate but not AMPA. *Nature (Lond.)* **351**:745–748 (1991).
15. Moriyoshi, K., *et al.* Molecular cloning and characterization of the rat NMDA receptor. *Nature (Lond.)* **354**:31–37 (1991).
16. Herb, A., *et al.* The KA-2 subunit of excitatory amino acid receptors shows widespread expression in brain and forms ion channels with distantly related subunits. *Neuron* **8**:775–785 (1992).
17. Meguro, H., *et al.* Functional characterization of a heteromeric NMDA receptor channel expressed from cloned cDNAs. *Nature (Lond.)* **357**:70–74 (1992).
18. Monyer, H., *et al.* Heteromeric NMDA receptors: molecular and functional distinction of subtypes. *Science (Washington D. C.)* **256**:1217–1221 (1992).
19. Sommer, B., and P. Seeburg. Glutamate receptor channels: novel properties and new clones. *Trends Pharmacol. Sci.* **13**:291–296 (1992).
20. Hughes, T., I. Hermans-Borgmeyer, and S. Heinemann. Differential expression of glutamate receptor genes (GluR1–5) in the rat retina. *Visual Neurol.* **8**:49–55 (1992).
21. Sommer, B., M. Kohler, R. Sprengel, and P. Seeburg. RNA-editing in brain controls a determinant of ion flow in glutamate-gated channels. *Cell* **67**:11–19 (1991).
22. Hollmann, M., M. Hartley, and S. Heinemann. Ca^{2+} permeability of KA- and AMPA-gated glutamate receptor channels depends on subunit composition. *Science (Washington D. C.)* **252**:851–853 (1991).
23. Edwards, F., A. Konnerth, B. Sakmann, and B. Takahashi. A thin slice preparation for patch clamp recordings from neurones of the mammalian central nervous system. *Pfluegers Arch.* **414**:600–612 (1989).
24. Mackler, S. A., B. Brooks, and J. Eberwine. Stimulus-induced coordinate changes in mRNA abundance in single post-synaptic hippocampal CA1 neurons. *Neuron* **9**:539–548 (1992).

25. VanGelder, R., *et al.* Amplified RNA (aRNA) synthesized from limited quantities of heterogeneous cDNA. *Proc. Natl. Acad. Sci. USA* **87**:1663–1667 (1990).
26. Eberwine, J., *et al.* Analysis of gene expression in single live neurons. *Proc. Natl. Acad. Sci. USA* **89**:3010–3015 (1992).
27. Eberwine, J., C. Spencer, K. Miyashiro, S. Mackler, and R. Finnell. cDNA synthesis *in situ*: methods and applications. *Methods Enzymol.* **216**:80–100 (1992).
28. Raff, M., *et al.* Cell-type-specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. *Brain Res.* **174**:283–308 (1979).
29. Saiki, R., *et al.* Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science (Washington D. C.)* **239**:487–491 (1988).
30. Surmeier, J., J. Eberwine, C. Wilson, Y. Cao, A. Stefani, and S. Kitai. Cellular and molecular evidence for the co-localization of dopamine receptor subtypes in acutely-isolated rat striatonigral neurons. *Proc. Natl. Acad. Sci. USA* **89**:10178–10182 (1992).
31. Lambolez, B., E. Audinat, P. Bochet, F. Crepel, and J. Rossier. AMPA receptor subunits expressed by single Purkinje cells. *Neuron* **9**:247–258 (1992).

Send reprint requests to: James H. Eberwine, Department of Pharmacology, University of Pennsylvania Medical School, Philadelphia, PA 19104–6084.