The structure of glutamate receptor–channel (GluR) subunits has recently been shown to differ from that of other ligand-gated channels and to contain a voltage-gated channel-like pore-forming motif. The view that the structure of GluR complexes is similar to the pentameric structure of other ligand-gated channels was questioned here. Studies of the response properties of the GluR1 subunit of the AMPA subtype of GluRs, co-expressed in *Xenopus* oocytes with its L646A mutant, which differs only by a greatly reduced sensitivity to quisqualate, provide new evidence suggesting that the GluR1 homomeric receptor channel has a tetrameric structure.

**Key words:** AMPA; cRNA; Glutamate receptor; Subunit stoichiometry; Tetramer; *Xenopus* oocytes

### Materials and Methods

**Oocyte expression:** Mutagenesis and oocyte expression were carried out as described previously. For over-expression of WT or mutant GluR1 together with GluR2, WT or mutant GluR1 cRNAs transcribed from pBTG (a vector which gives very high translation rates) were injected together with GluR2 cRNA transcribed from a pSK– (Stratagene, a vector which gives regular expression levels).

**Determination of subunit stoichiometry:** Two approaches were used: the first was based on the calculation of expected WT fractions, the second on the formalism developed by MacKinnon. For the former, theoretical curves (Fig. 3) were derived from a binomial distribution:

\[
F_i = \frac{n!}{i! \cdot (n - i)!} \cdot (f_{\text{mut}})^i \cdot (f_{\text{wt}})^{(n-i)}
\]

where \(F_i\) is the fraction of receptor complexes having \(i\) mutant subunits, \(n\) is the number of subunits in the complex, \(f_{\text{mut}}\) is the fraction of mutant subunits within the total population and \(f_{\text{wt}}\) (which equals \(1 - f_{\text{mut}}\)) is the fraction of WT subunits. On the basis of the similarity between the levels of expression of functional WT and mutant receptors when expressed as homooligomers or as heteromers with GluR2 (Fig. 1A,B), it is assumed that the co-injection of WT and mutant cRNA into oocytes will result in the formation of hybrid receptors with \(f_{\text{wt}}/f_{\text{mut}}\) ratios equal to the WT/mutant cRNA ratios, allowing the use of cRNA ratios in this calculation. When suggesting that the presence of one WT subunit in the hybrid receptor complexes is enough to confer a WT-like high QA response, the expression of GluR1 subunits was compared to that of GluR1 mutants and the cRNA ratios were used in the calculation.

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**A tetrameric subunit stoichiometry for a glutamate receptor–channel complex**

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sensitivity, we assume all the hybrid receptor complexes behave like WT complexes and thus
\[ F(x) = 1 - F_n. \]
When assuming that at least two WT subunits are needed to confer WT-like high QA sensitivity, the expected relationship is
\[ F(x) = 1 - [F_n + F(x - 1)]. \]
In both cases, the expected \( F(x) \) values (for the trimeric to hexameric stoichiometries) were calculated for the specific experimental \( f_{mix} \) and interpolation was used to generate expected \( F(x) \) curves.

For the second approach, we used the equation developed by MacKinnon? \n\[ \ln(U_{mix}/U_{max}) = n - 1/\ln(f_{max}) \ln(1 - R/U_{mix}). \]

The responses of each oocyte were calculated for \( \ln(U_{mix}/U_{max})/\ln(f_{max}) \), where \( U_{mix} \) is the fractional response of the oocyte expressing a mixed receptor population at a given QA concentration, \( U_{max} \) is the average fractional response of oocytes expressing only mutant receptors at this particular QA concentration, and \( f_{max} \) is as above. At each QA concentration, these values were averaged for all the analyzed oocytes and plotted against the QA concentration. In order to calculate the expected theoretical values, the right hand side of the above equation was calculated:
\[ n - 1/\ln(f_{max}) \times \ln(1 - R/U_{mix}) \]
where \( n \) is the proposed subunit stoichiometry. \( f_{max} \) is as defined above (weighted according to the number of oocytes from each combination ratio used here); \( R = (F_0 + F_1 + \ldots + F_{n - 1}) \times IC\text{\textsubscript{IC50}/}[IC\text{\textsubscript{IC50} + [QA]}] \) where \( F_0 + F_1 + \ldots + F_{n - 1} \) is the combined fraction of all the species containing at least one WT subunit according to a binomial distribution (calculated as described above) and \( IC\text{\textsubscript{IC50}} \) is the QA IC\textsubscript{IC50} value of WT GluR1 corrected according to the KA concentration used.\(^{10,12} \) Although not simple or pure, the interaction between KA and QA can be closely described as competitive,\(^{10,12} \) allowing the calculation of \( IC\text{\textsubscript{IC50}} \) as described by Patneau and Mayer.\(^{10} \)

\[ IC\text{\textsubscript{IC50}} = R + F_n \times IC\text{\textsubscript{IC50}}/([IC\text{\textsubscript{IC50}} + [QA]]) \]
where \( F_n \) is the fraction of complexes containing only mutant subunits and \( IC\text{\textsubscript{IC50}} \) is the corrected QA IC\textsubscript{IC50} value of the mutant subunit. When [QA] is high, \( R \) approaches 0 and the calculated value approaches \( n \).

**Results and Discussion**

Using site-directed mutagenesis, we have recently identified some of the amino acid residues present at the agonist binding site of GluR1.\(^8 \) Monitoring the expression of homomeric GluR1 channels in *Xenopus* oocytes by current measurement under voltage clamp, we have observed that the L646A mutant has most of the properties of the WT GluR1: for equal amounts of injected cRNA, its levels of expression are very similar to those of WT GluR1 (Fig. 1A,B); the WT and L646A GluR1 mutant display the same apparent affinity to KA (Fig. 1C); the KA responses of WT and L646A GluR1 mutant are potentiated to the same extent by saturating concentrations of cyclothiazide (CYZ), a desensitization-attenuating drug,\(^14,15 \) indicating that prior to the application of CYZ the equilibrium between the open and desensitized states of KA-activated WT and mutant receptors was the same.\(^4 \) Nonetheless, the L646A GluR1 mutant differs from the WT in its QA-induced desensitization properties. These could not be readily observed upon application of the desensitizing agonist QA alone since, in both WT and mutant receptors, hardly quantifiable steady state currents (< 10 nA) were observed. However, a large difference in the sensitivity to QA could be detected, in

![Image](image_url)
A tetrameric stoichiometry for an AMPA-R

the presence of CYZ, as a shift in QA EC_{50} values and a change in the steady state/peak currents ratios or, in the absence of CYZ, in the dose-dependencies of the inhibition by QA of KA-evoked currents, which were found to differ between WT and mutant receptors by a factor of ~100 (Fig. 1D).

The combination of features of WT and of the L646A GluR1 described above provided the necessary conditions to establish the subunit stoichiometry of GluR1 receptor channels on the basis of the rules of a binomial distribution. These require that the WT and mutant L646A GluR1 channel subunits express equally and form mixed oligomeric complexes at random, as demonstrated in Fig. 1A and B.

We therefore co-injected oocytes with various weight ratios of cRNAs encoding the L646A GluR1 mutant and WT GluR1 and studied the QA sensitivity of the expressed channel complexes (Fig. 2). For most of these combinations, a biphasic inhibition can be clearly distinguished. A very good fit of these data could be obtained by using the sum of two logistic equations, revealing the contributions of two receptor populations with distinct IC_{50} values of 1.4 ± 0.3 μM and 81 ± 33 μM QA. As these IC_{50} values are quite similar to those of homomeric WT and L646A mutant GluR1 (1.1 ± 0.3 μM and 99 ± 15 μM QA, respectively, referred hereafter as the parental affinities), the latter values were used in fact to fit the data and yield, as software output, the calculated contribution of each parental population to the hybrid receptor response (solid curve lines in Fig. 2). Attempts to fit the data using free parameters describing an additional intermediate affinity resulted in strong shifts of the affinities attributed to the homomeric WT and L646A mutant GluR1 and in inconsistency between the anticipated contribution of parental and intermediate affinities to the total response, as expected from a binomial distribution according to the fractions of cRNA used, and the actual contribution of each affinity-type, as calculated from the data. We therefore conclude that only the parental QA sensitivities are displayed by the hybrid receptor populations, with no expression of intermediate sensitivities.

Examining the contributions of each receptor population to the observed responses, the relative contribution of the expressed WT phenotype was larger then its original proportion in the cRNA injected (e.g. a 1:5 WT:mutant cRNA ratio exhibits an approximately equal contribution of the parental phenotypes). This indicates that hybrid receptor complexes containing WT and mutant subunits do indeed form and that, in these complexes, the WT high sensitivity to QA is dominant. Figure 3 compares the observed relationship between the proportions of WT cRNA injected and the fractions of receptor populations displaying the WT phenotype to the corresponding theoretical curves expected from the binomial distributions of complexes formed.
by the assembly at random of WT and mutant subunits into trimers and up to hexamers. Inspection of Fig. 3 reveals that, regardless of the suggested stoichiometry, the experimental data clearly align with the theoretical curves calculated for the case in which the presence of a single WT subunit in the hybrid receptor has a dominant effect and is enough to account for the high QA sensitivity. Therefore we conclude that the interaction of QA with only one of the receptor subunits is the necessary and sufficient condition for causing the desensitization of a KA-activated oligomeric channel complex. Analysis of the average misfit between the observed WT fractions and the various theoretical values suggests that the data fit best with a tetrameric stoichiometry model.

To determine in an additional fashion the subunit stoichiometry of the GluR1 receptor channel, we further used the sets of equations developed by MacKinnon for the analysis of the subunit stoichiometry of the Shaker potassium channels.9 Figure 4 presents the results of this analysis for pooled and averaged data from four different combinations of cRNA, along with the theoretical curves for stoichiometries of 3, 4 and 5. At high QA concentrations the curve reaches asymptotically a value of n = 4. χ² analysis of the set of data, comparing the values expected according to each stoichiometry to the actual values observed for the various QA concentrations, indicates that the tetrameric stoichiometry is ~900 and ~3000 times more likely than the pentamic and trimeric stoichiometries, respectively. We therefore suggest that the GluR1 receptor complex expressed in Xenopus oocytes is composed of four subunits.

The limitation common to both methods of analysis is the existence of so far undetected differences between the WT GluR1 and L646A GluR1 mutant, which may lead to their unequal functional expression. This will bias the binomial distribution and preclude the present stoichiometry analysis. In this respect, our present conclusion will remain valid at least as long as the true $f_{\text{mut}}$ values do not differ significantly from the set $f_{\text{mut}}$ values. The results presented in Fig. 1A, B and C suggest that this condition is likely to be fulfilled. Therefore, on the basis of the results of the two methods used here to analyze subunit stoichiometry, we conclude that a tetrameric stoichiometry is more likely than either the trimeric, pentamic or hexameric stoichiometry. We limit our conclusion to the most probable and simple stoichiometry model but cannot overrule the possible fitting of our data with more complex models.

Beyond the confines of the above mentioned restrictions, we tend to assume that the tetrameric stoichiometry presently suggested for the GluR1 subtype of AMPA-Rs is a feature common to all GluRs, including those expressed in a natural environment. Although some of the biochemical studies that dealt with the determination of the molecular weight of GluRs have favored a pentamic structure, the published data do not rule out a core tetrameric structure, while other even suggest it if considered in conjunction with the proposition that GluRs may interact in situ with other proteins which may remain associated with GluRs when the latter are solubilized with weak detergents.5,6,17,18 Studies of GluR channel properties either do not overrule2 or suggest19,20 a tetrameric structure.

Recently, two papers that deal directly with the subunit stoichiometry of AMPA-Rs have reached contradictory conclusions. Ferrer-Montiel and Montal used two GluR1 mutants with amino acid substitutions at the channel pore region and the set of equations developed by MacKinnon9 to suggest a pentamic stoichiometry for GluR1 receptor channels.21 However, the mutants tested were not shown to be expressed at the same levels since the amplitudes of their current responses varied up to 10-fold and no data on their distribution and mean values were presented. Furthermore, the channel properties of the two receptor species significantly differ in their bivalent ion permeabilities. This difference leads, according to Lewis’s current equation,22 to unequal amplitudes of current responses recorded from receptor-channels expressed to the same protein level, which may then be further amplified in the oocyte by the secondary activation of Ca²⁺-dependent Cl⁻ channels (for comparison, see recording conditions used by Hume et al.23). Under such circumstances, the fractions of currents recorded...
in a mixing experiment are not directly proportional to the ratios of injected cRNAs, leading to an inaccurate estimation of the fraction of the toxin-insensitive species and of the receptor/channel stoichiometry. In the other paper, Wu et al.\textsuperscript{24} present a variety of compelling biochemical lines of evidence to support the conclusion that AMPA-Rs are formed by a core complex of four subunits.

Conclusion

The question of the stoichiometry of GluRs has been a subject of controversy. We believe that previous biochemical and functional studies\textsuperscript{1,7,19,20} and the recent study by Wu et al.\textsuperscript{24}, together with our present data, provide an increasingly strong progression of corroborating evidence for the tetrameric stoichiometry of AMPA-Rs. The presently proposed tetrameric stoichiometry of GluRs provides an additional support to the concept of the existence of an evolutionary link between GluRs and voltage-gated channels.

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References
