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Philippe BEhe, Peter Stern, David J. A. Wyllie, Mohammed Nassar, Ralf Schoepfer, David Colquhoun

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Determination of NMDA NR1 subunit copy number in recombinant NMDA receptors

PHILIPPE BÉHÉ, PETER STERN, DAVID J. A. WYLLIE, MOHAMMED NASSAR, RALF SCHOEPFER AND DAVID COLQUHOUN*

Department of Pharmacology and Wellcome Laboratory for Molecular Pharmacology, University College London, Gower Street, London WC1E 6BT, U.K.

SUMMARY

Co-expression of wild-type and mutated NMDA NR1(N598R) subunits in *Xenopus* oocytes has been used to determine the stoichiometry of the NMDA receptor-channel. When expressed together, wild-type NR2A and mutant NR1(N598R) subunits produced channels with a main conductance of 2.6 pS and a sublevel of 1.2 pS. These conductances were clearly different from those obtained from wild-type NR1 and wild-type NR2A channels which gave characteristic 50 pS events with a 40 pS sublevel. When wild-type and mutant NR1 subunits were co-expressed together with NR2A subunits a different channel type with a main conductance of 15.2 pS and a sublevel of 11.4 pS was obtained, as well as the 'all wild-type' and 'all mutant' channels described above. These results indicate that there are likely to be two copies of the NR1 subunit in each NMDA receptor complex.

1. INTRODUCTION

Fast excitatory neurotransmission in the mammalian central nervous system (CNS) is mediated primarily by the amino acid glutamate acting on two classes of ion channel-linked receptors, namely *N*-methyl-D-aspartate (NMDA) receptors and non-NMDA receptors. In addition to their role in synaptic transmission, NMDA receptors have been the focus of much research because of their modulation by many endogenous compounds and second messenger systems, because of their probable involvement in CNS development and in changes in synaptic efficacy, and because of their role in neurotoxicity and disease states (for a review see McBain & Mayer 1994).

Two kinds of NMDA receptor subunits, the NR1 and NR2 subunits, have been cloned so far (Moriyoshi *et al.* 1991; Meguro *et al.* 1992; Monyer *et al.* 1992; Ishii *et al.* 1993). The NR1 subunit, which is found in seven different splice variants (Hollmann *et al.* 1993), is ubiquitously expressed throughout the CNS and appears to be the fundamental subunit required for NMDA channel function. In contrast, the NR2A, NR2B, NR2C, and NR2D subunits each show more localized expression in different brain regions. Recombinant coexpression of NR1 with any of the NR2 subunits yields large agonist-gated cation currents, whereas homomeric expression yields no current at all with NR2 subunits, or very small currents in the case of NR1 subunits. Thus the most efficient form of the NMDA receptor is a heterooligomer (for reviews see Nakanishi 1992; Seeburg 1993). Despite the recent intense interest in NMDA receptors, the subunit stoichiometry of these channels remains unknown. Subunit composition has been studied extensively in the gene family that includes the receptors for acetylcholine (AChR), γ-amino-butyric acid (GABA-R) and glycine (Gly-R) (for example: Langosch *et al.* 1988; Anand *et al.* 1991; Cooper *et al.* 1991; Unwin 1993). However, subunits for both NMDA and non-NMDA receptors belong to a gene family that is clearly different from the AChR/GABA-R/Gly-R family. Therefore, stoichiometry of NMDA receptors cannot be inferred from that of AChRs. An approach similar to that used by Cooper *et al.* (1991) to elucidate the stoichiometry of chick neuronal nicotinic receptors can be used to determine the copy number of NR1 subunits in the NMDA receptor-channel complex. This method uses co-expression of wild-type and mutant subunits, where the mutant subunit confers an altered single-channel conductance. Receptor channel complexes having both mutant and wild-type subunits incorporated are expected to result in intermediate single-channel conductances. By counting the number of distinct molecular species formed under these circumstances, we were able to determine the number of NR1 subunits contained in a NMDA receptor-channel complex.

2. METHODS

(*a*) *Preparation and expression of cRNA*

The cDNAs coding for the wild-type rat NR1a subunit (splice variant naming as in Hollmann *et al.*

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1993) and the NR2A subunit have been described in Monyer *et al.* (1992), and the NR1(N598R) mutant was described by Burnashev *et al.* 1992. cRNA was obtained from high-expressing constructs (Burnashev *et al.* 1992; Ruppersberg *et al.* 1993; Schoepfer *et al.* 1994) bearing the appropriate insert in a pSP64T (Krieg & Melton 1984) derived vector backbone using the Promega (Madison WI) RiboMAX RNA synthesis kit. Reactions were supplemented with 3.75 mM capping nucleotide m7G(5′)ppp(5′)G (Pharmacia) in the presence of 1.6 mM GTP. The NR1 wild-type and mutant constructs differ only in the mutated area, all other parts of the constructs are identical. For coinjection of wild-type and mutant NR1, a nominal ratio of 1:1 of the two cRNAs was used.

Xenopus laevis were anaesthetized by immersion in a 0.1% solution of tricaine (3-amino benzoic acid ethyl ester); their ovarian lobes were removed and placed in a modified Barth's solution. After mechanical dissociation, oocytes were treated with collagenase (Type 1A, Sigma, 1 mg ml^{-1} for 1 h). The follicular membrane was removed and oocytes were injected with cRNA. Oocytes were placed in modified Barth's solution containing 30 μM D-(−)-2-amino-5-phosphonovaleric acid (APV) and kept at 19 °C. Patch recordings were usually made 3–7 days after injection.

(*b*) *Solutions*

The modified Barth's solution contained (in millimoles per litre): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 15 Tris/HCl, 0.33 $Ca(NO_3)_2$, 0.44 $CaCl_2$, 0.82 $MgCl_2$, 50 U ml^{-1} penicillin and 50 U ml^{-1} streptomycin. Oocytes were screened for responses to 10 μM glutamate and 10 μM glycine in a solution containing (in millimoles per litre): 115 NaCl, 2.5 KCl, 1.8 $BaCl_2$, 10 HEPES. Intracellular microelectrodes (0.2–0.8 MΩ) were filled with 3 M KCl. Patch-pipettes were filled with an internal solution composed of (in millimoles per litre): 2.5 NaCl, 147 gluconolactone, 147 NaOH, 10 HEPES, 11 EGTA (pH 7.4 with NaOH). The external solution contained (in millimoles per litre): 125 NaCl, 2.5 KCl, 1 $CaCl_2$, 26 $NaHCO_3$, 1.25 $NaHPO_4$, 25 glucose (pH 7.4 when bubbled with 95% O_2 / 5% CO_2). The following drugs were used: L-glutamate, glycine (Fluka), D-APV (Tocris) and the salts used to prepare external and internal solutions were of high purity (Aristar grade, BDH).

(*c*) *Data acquisition and analysis*

The vitelline membrane of the oocyte was removed immediately before patch-clamp recording (Methfessel *et al.* 1986). Single-channel currents were recorded in the outside-out patch configuration (Hamill *et al.* 1981) using an Axopatch 1D amplifier (Axon Instruments). Patch-pipettes were made from thick-walled hard (borosilicate) glass (I. D. 0.86 mm O.D. 1.5 mm, Clark Electromedical). The ends of patch-pipettes were coated with Sylgard (Dow-Corning) to reduce their capacitance, their tips were fire-polished and they had resistances of 15–25 MΩ. To prevent exposing the rest of the oocyte to glutamate-containing solutions, patches were moved into a compartment of the recording chamber that could be physically isolated and independently perfused from the oocyte-containing chamber. Patches were held at −100 mV.

Single-channel currents were filtered at 20 kHz and stored on digital audio tape (DTR 1200, Biologic) for subsequent 'off-line' analysis. For single-channel analysis currents were replayed from tape and, depending on the receptor combination to be analysed, were filtered at either 200, 500 or 2000 Hz (8-pole Bessel filter, −3 dB) and continuously digitized at 5, 10 or 20 kHz respectively (CED 1401 plus, Cambridge Electronic Design). The amplitudes and durations of channel openings were fitted simultaneously with the SCAN program (see Colquhoun & Sigworth 1995).

Histograms of fitted single-channel amplitudes were constructed from events that had durations longer than 2.5 T_r (or 2.0 T_r), where T_r is the filter rise time; i.e. amplitudes were used only from those events that were long enough to reach at least 99.8% (or 98.8%) of their full amplitude (except for figure 4*c*). Such histograms were fitted with a mixture of several Gaussian components using the method of maximum likelihood, with the EKDIST program. Amplitude stability plots of events with durations of at least 2.5 T_r (or 2.0 T_r) were also constructed in order to determine if there were any temporal changes in the amplitude of events during the course of an experiment (see Colquhoun & Sigworth 1995). Results were used only when the amplitudes were stable throughout.

3. RESULTS

The mutation which we refer to as NR1(N598R) replaces an asparagine residue with an arginine residue in the region that is thought to form a hairpin loop in the recently proposed model of glutamate receptor topology (see Hollmann *et al.* 1994; Wo & Oswald 1994. 1995; Bennett & Dingledine 1995). This residue has been shown to control the permeability of the NMDA receptor to Ca^{2+} and also is the site responsible for the voltage-dependent block of the receptor by Mg^{2+} (Burnashev *et al.* 1992; Mori *et al.* 1992). The N598R mutation has been reported to reduce the single-channel conductance of the NMDA receptor so much that single-channel openings cannot be resolved (Ruppersberg *et al.* 1993). However we have found that, with appropriate filtering, very small openings can be resolved and measured (figure 2). We chose, therefore, to use this mutant because it provides a very large separation in the amplitude distributions of single-channel currents from receptor complexes incorporating only 'mutant' or only 'wild-type' NR1 subunits.

(*a*) *Whole-cell responses*

Oocytes were tested for their responsiveness to 10 μM glutamate and 10 μM glycine prior to patch-clamp experiments. The mean currents evoked in oocytes that were used for patching were 5.5, 6 and 4.7 μA for wild-

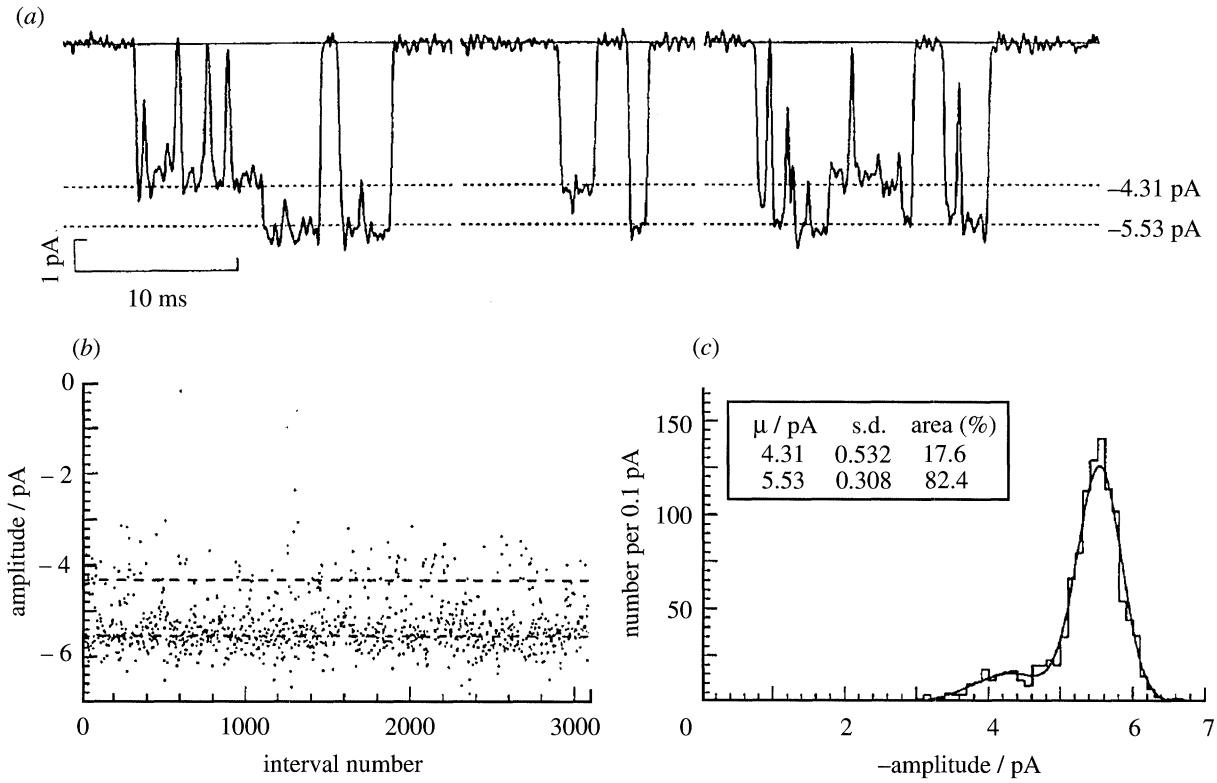

Figure 1. Single-channel currents, amplitude stability plot and amplitude distribution for channels in an outside-out patch obtained from an oocyte injected with cRNA coding for NR1 and NR2A subunits. Glutamate concentration was 200 nM (with 10 μM glycine). (*a*) Single-channel currents recorded at −100 mV and filtered at 2 kHz (−3 dB). The two amplitude levels indicated were obtained from the amplitude histogram in (*c*). Direct transitions between the two conductance levels are clearly present in the traces on the left and right. (*b*) Amplitude stability plot for the experiment shown in the histogram in (*c*). The dashed lines indicate the mean amplitudes obtained from the fit shown in (*c*). (*c*) Amplitude distribution of 1173 events which had durations greater than 332 μs (2.0 T_r). The distribution is fitted by two Gaussian components, with means of 4.31 pA (17.6%) and 5.53 pA (82.4%).

type, all-mutant and mixed subunits respectively, when oocytes were screened 3 days after injection.

(*b*) *Single-channel currents with wild-type NR1 and wild-type NR2A subunits*

Application of 20–200 nM glutamate, in the presence of 10 μM glycine, to outside-out patches excised from oocytes injected with cRNA encoding NR1 (wild-type) and NR2A receptor subunits gave single-channel currents with a main conductance of around 50 pS and a sublevel of around 40 pS. These are characteristic for this subunit combination in 1 mM external Ca^{2+} (Stern *et al.* 1992), as well as being similar to those seen in many native NMDA receptors. Figure 1*a* shows two typical bursts of NMDA channel activity and also illustrates examples of direct transitions between the 40 and 50 pS conductance levels. The amplitude stability plot in figure 1*b* indicates that during the course of the experiment there was little variation of the amplitude of the currents recorded; the dotted lines indicate the amplitude levels of the two Gaussian components fitted to the amplitude histogram in figure 1*c*. The means for this experiment were 4.3±0.5 pA (17.6%) and 5.5±0.3 pA (82.4%). The average amplitudes and areas for 'all wild-type' patches examined are given in table 1.

The fact that many of the '40 pS' openings arise by direct transitions from the main '50 pS' level, and that the frequencies of the two levels, and of transitions between them, are similar from patch to patch, has given rise to the view that this behaviour of the wild-type channel arises from a single molecular species which can open to both conductance levels (see §4).

(*c*) *Single-channel currents with mutant NR1 and wild-type NR2A subunits*

Single-channel currents resulting from the opening of NR1(N598R)-NR2A channels were very small, but, luckily, their mean lifetime was sufficiently long for heavy filtering (usually 200 Hz, −3 dB) to make many of them clearly visible without attenuation of their amplitudes. At −100 mV the channels showed a main level at around 0.25 pA with a sublevel at around 0.12 pA. Figure 2*a* shows three typical openings of this 'all mutant' channel. The 0.12 pA sublevel is clearly visible. The amplitude stability plot in figure 2*b* plots the amplitude of each event with a duration longer than 2.0 T_r from an experiment containing 313 such events. Figure 2*c* illustrates the amplitude histograms for this experiment. The distribution was well fitted by two Gaussian components. The mean values for the amplitudes of channels (and their areas) for patches injected with the NR1(N598R) subunit are given in table 1. Again we feel confident that, as with 'wild-

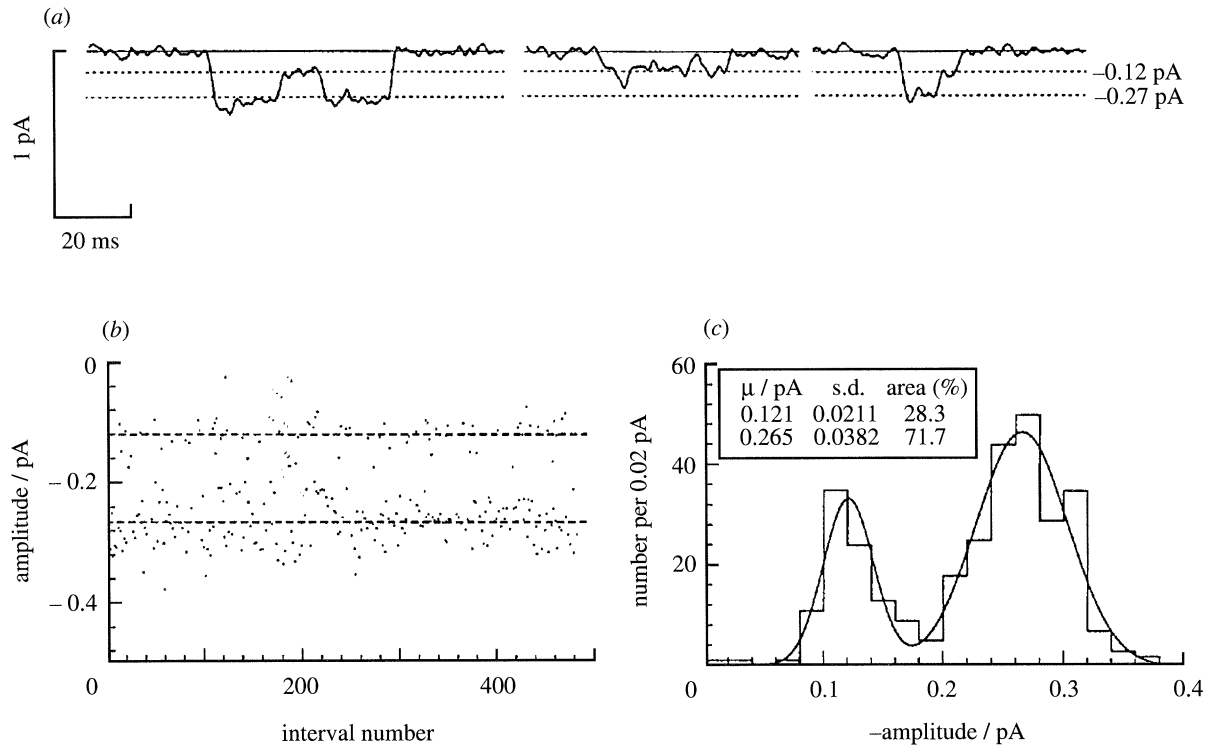

Figure 2. Single-channel currents, amplitude stability plot and amplitude distribution for channels in an outside-out patch obtained from an oocyte injected with cRNA coding for NR1(N598R) and NR2A subunits. Glutamate concentration was 200 nM (with 10 μM Glycine). (*a*) Single-channel currents recorded at −100 mV and filtered at 200 Hz (−3 dB). The two amplitude levels indicated were obtained from the amplitude histogram in (*b*). Direct transitions between the two conductance levels are evident. (*b*) Amplitude stability plot for the experiment shown in the histogram in (*c*). The dashed lines indicate the mean amplitudes obtained from the fit shown in (*c*). (*c*) Amplitude distribution for events with durations greater than 3.32 ms (2.0 T_r). The histogram contains 313 events and is fitted by two Gaussian components, with means 0.12 pA (28.3%) and 0.26 pA (71.7%).

type' channels, the lower amplitude channel is indeed a sub-conductance of the main level, so the two levels represent a single molecular species (see §4). Thus it seems likely that injection of oocytes with either NR1 or NR1(N598R) subunits each produced a substantially homogeneous population of channels which represent a single preferred subunit association between NR1a and NR2 subunits.

(*d*) *Single-channel currents from co-injection of wild-type and mutant NR1 and wild-type NR2A subunits*

We next injected oocytes with a mixture of cRNAs encoding both NR1 and NR1(N598R) subunits, together with cRNA for NR2A. When both types of subunit are available for receptor assembly one would expect, under the simplest hypothesis (see §4), to see $(n+1)$ distinguishable molecular species, where n is the copy number of NR1 subunits present in the NMDA channel complex. Therefore, if the number of molecular species that are generated can be inferred from observations of channels in patches excised from oocytes injected with the mixture of the two NR1 subunits, we should be able to determine the number of NR1 subunits contained in the NMDA channel complex.

Outside-out patches from oocytes injected with the two types of NR1 subunit gave, as expected, single-channels that were the same as those already observed for the 'all wild-type' and the 'all mutant' injections. In addition, patches also gave single-channel openings of a new type with a main amplitude of around 1.2 pA. Typical openings of this additional channel are shown in figure 3*a*. The stability plot and distribution of their amplitudes are shown in figure 3*b* and *c* respectively.

Figure 4 illustrates the whole population of channels seen in the same patch. Figure 4*a* shows (to the same scale) representative single-channel currents for each of the three conductance patterns that were observed. The amplitude stability plot in figure 4*b* clearly illustrates the presence of channels with amplitudes not observed in either the 'all wild-type' or 'all mutant' patches. The distributions of fitted amplitudes for the same patch (figure 4*c*), and for a different patch (figure 4*d*), similarly show the presence of two additional peaks in the histogram at around 1.2 pA and 1.6 pA. The histogram in figure 4*d* is fitted with six Gaussian components, whereas that in figure 4*c* is fitted by the sum of five Gaussian components, there being insufficient 'wild-type' events in this case to allow for the clear resolution of both the '50 pS' and '40 pS' components.

The two additional peaks seen in these distributions originate, almost certainly, from a single molecular species that displays two conductance levels, because

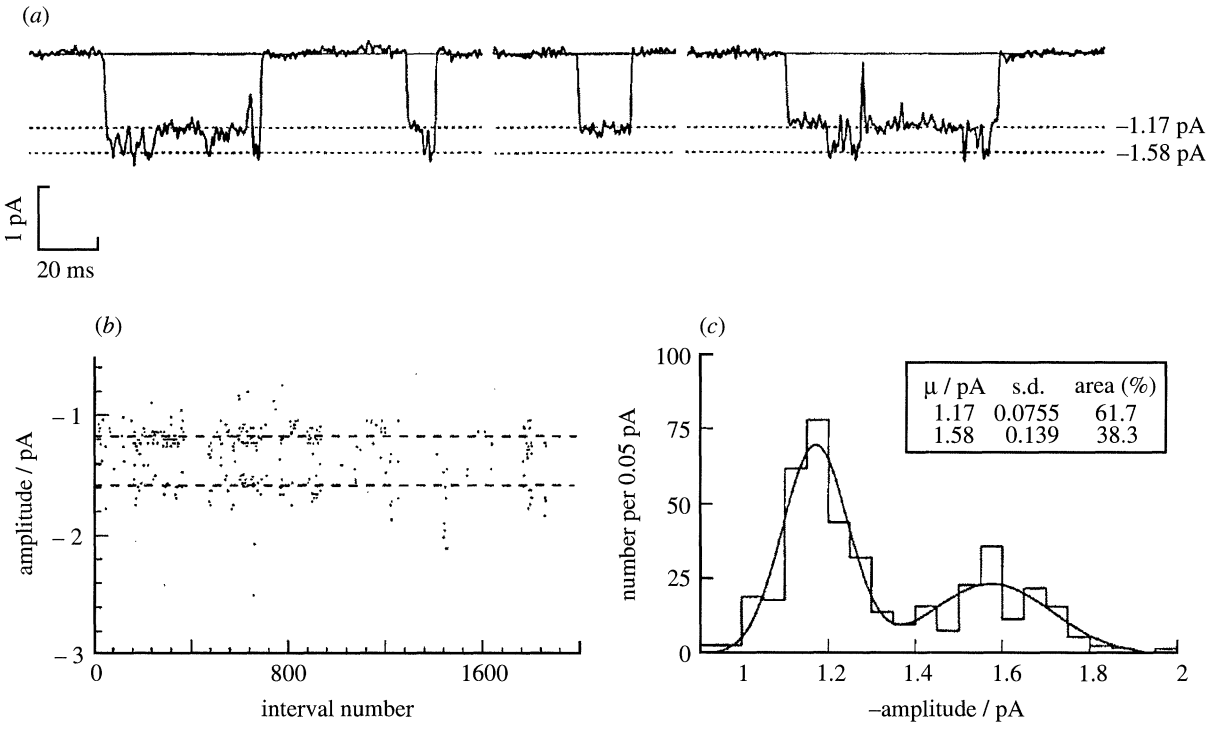

Figure 3. Single-channel currents, amplitude stability plot and amplitude distribution for a subpopulation of the channels activated by 200 nM glutamate and 10 μM glycine in an outside-out patch obtained from an oocyte injected with cRNA coding for NR1, NR1(N598R), and NR2A subunits. The total channel population in the same patch is described by figure 4*a–c*. (*a*) Single channels currents recorded at −100 mV and filtered at 500 Hz (−3 dB), representative of the events which appear in the amplitude histogram shown in (*c*). The two amplitude levels indicated were obtained from the fit of that histogram. Direct transitions between the two conductance levels are evident. (*b*) Amplitude stability plot for the events that appear in the histogram in (*c*). The dashed lines indicate the mean amplitudes obtained from the fit of that histogram. (*c*) Amplitude distribution of 424 such events which had durations greater than 1.33 ms (2.0 T_r). The histogram is fitted with two Gaussian components with means 1.17 pA (61.7%) and 1.58 pA (38.3%).

there are frequent direct transitions between the 1.2 pA and the 1.6 pA levels (as in figure 3*a*), and because the frequency of both levels are similar from patch to patch. These openings are, we suggest, from channels that contain one wild-type and one mutant NR1 subunit. The 1.6 pA openings constitute a minor component, being both briefer and rarer (about 30% of area, see table 1) than openings to 1.2 pA. The facts that no channels with an amplitude of 0.4 pA were observed, and that direct transitions beetwen the 1.6 pA level and the shut level were common, rule out the possibility that the 1.6 pA level represented superposition of a second channel on the main 1.2 pA openings. Very rarely a third level at about 0.85 pA was seen (the arrows in figure 4*a* point at two such events). Because direct transitions between them and other levels were seen (figure 4*a*), we believe that they arise from the same molecular species that produces 1.2 and 1.6 pA openings. In no patches were these events sufficiently frequent to allow them to be fitted. We therefore used only two components to fit the amplitude distribution for the 'mixture' channel, although the third level does appear in some distributions (see arrow in figure 4*c*).

We have examined ten such 'mixture' patches and the mean amplitudes and areas are given in table 1. As each of the three channel types seen in these patches represents distinct channel complexes we fitted the main conductance and its associated sublevel as separate distributions, therefore the areas for each channel type add to 1. Of the ten patches examined, eight contained all three channel types, while the remaining two did not contain any detectable wild-type channels. In no patches did we observe any channel type that did not correspond to one of the three channel types documented above.

One might expect the ratio between the number of observations of each channel type to be influenced by the ratio in which the cRNAs for wild-type and mutant NR1 subunits were injected. However, the fluctuations in the probability of being open for individual NMDA channels (e.g. Gibb & Colquhoun 1992; Stern *et al.* 1992) are large, and the number of channels per patch is probably small so there will be large random variability in the number and type of channels in each patch. Therefore it is not surprising that we have observed a highly variable proportion of each channel type from patch to patch with a constant ratio of injected RNA (nominally 1:1). A comparison of the histograms in figure 4*c*, *d*, in which the proportion of wild-type channels varies from 11.4% to 66.4% illustrates this point. Under those conditions, additional variations induced by modifying the cRNA ratio are expected to be detectable only after averaging the proportions observed on a large number of patches for each ratio. We did not attempt to do this.

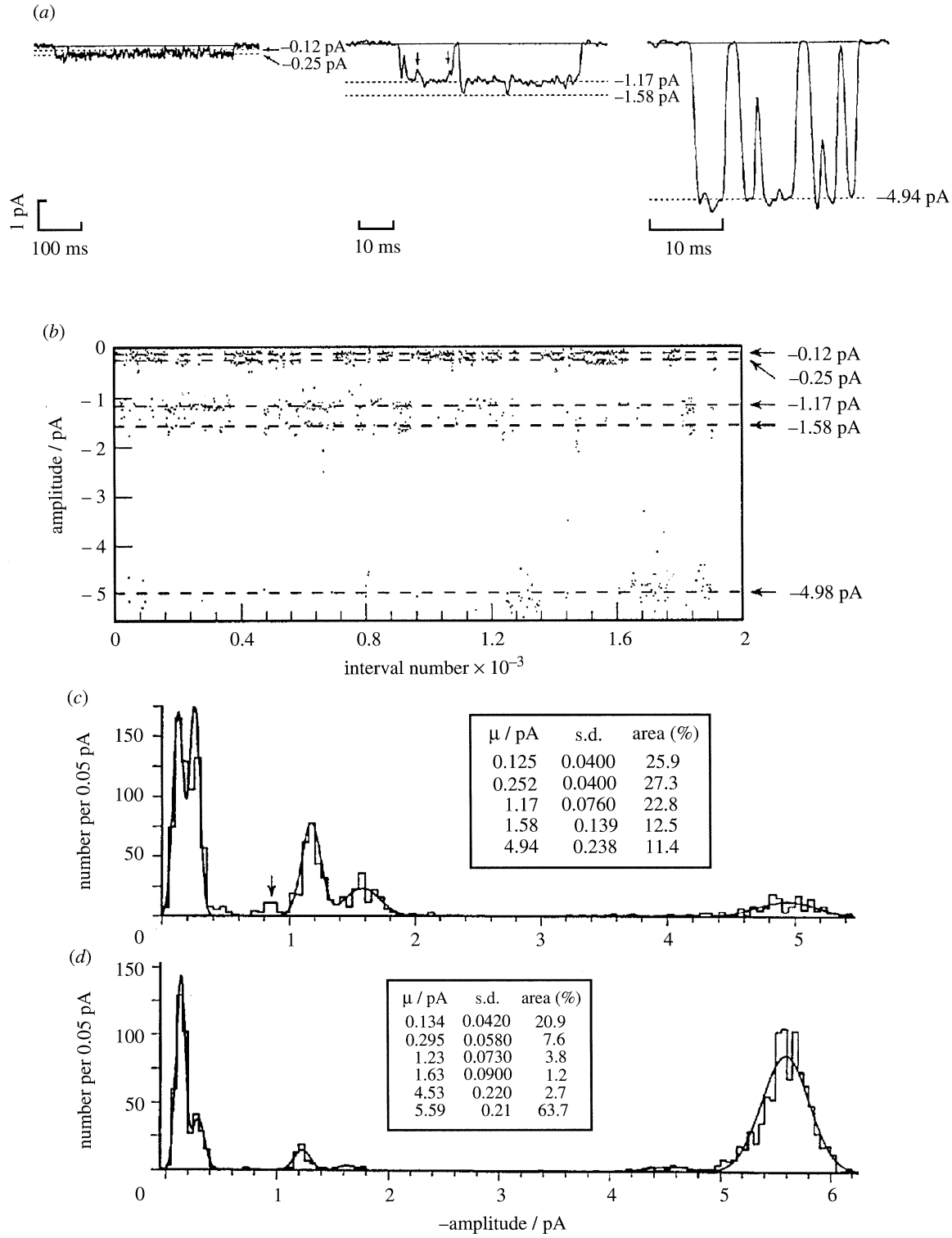

Figure 4. Single-channel currents, amplitude stability plot and amplitude distributions representative of channel populations in patches obtained from oocytes injected with cRNA coding for NR1 (wild-type), NR1(N598R) and NR2A subunits. (*a*) Single channel traces showing, from left to right, and on a common vertical scale, 'all-mutant', 'mixture' and 'all-wild-type' channels. All were recorded from the same patch, at −100 mV, with 300 nM glutamate and 10 μM glycine and filtered at 500 Hz (−3 dB). The dashed lines indicate the mean amplitudes obtained from the fit shown in (*c*). The arrows point at the rare events discussed in the text. (*b*) Amplitude stability plot for 1137 openings such as those shown in (*a*) but of durations greater than 1.33 ms (2.0 T_r). The dashed lines indicate the mean amplitudes obtained from the fit shown in (*c*). (*c*) Amplitude distribution of 1348 openings such as those shown in (*a*). In this case the distribution includes openings below 2 pA longer than 1.33 ms (2.0 T_r) and openings above 2 pA longer than 0.33 ms (0.5 T_r). The arrow points at the minor component discussed in the text. Data shown in (a), (b) and (c) and in figure 3 are all from the same patch. (d) Amplitude distribution of 1442 openings of duration greater than 1.33 ms (2.0 T_r) observed on another 'mixture' patch at −100 mV in the presence of 30 nM glutamate and 10 μM glycine.

Table 1. *Average single-channel current amplitudes and areas for receptor combinations NR1/NR2A, NR1(N598R)/NR2A and NR1/NR1(N598R)/NR2A*

(Single-channel amplitudes and areas were obtained from the fitting of multiple Gaussian components to distributions of events with durations greater than 2.5 T_r (or 2.0 T_r). The amplitudes (and areas) for the sublevel and main level for channels in either 'all wild-type' or 'all mutant' patches were not significantly different from the values obtained for these channels in oocytes injected with NR1, NR1(N598R) and NR2A subunits ($p > 0.1$). Values are given as means±s.e.m.; n indicates the number of patches.)

subunit combination	n	amplitude/pA (area %)	amplitude/pA (area %)	amplitude/pA (area %)	amplitude/pA (area %)	amplitude/pA (area %)	amplitude/pA (area %)
NR1/NR2A	4					4.26±0.12 (17.6±6.3)	5.30±0.12 (82.4±6.3)
NR1(N598R)/NR2A	7	0.12±0.003 (33.7±6.3)	0.26±0.01 (66.3±6.3)				
NR1/NR1(N598R)/ NR2A	10	0.11±0.005 (30.1±6.1)	0.25±0.01 (69.9±6.1)	1.16±0.02 (68.3±3.5	1.51±0.04 (31.7±3.5)	3.98±0.41 (9.3±4.6)	4.76±0.19 (90.7±4.6)

4. DISCUSSION

The results presented above provide strong evidence for the view that each NMDA receptor oligomer contains two NR1 subunits. The results are consistent with the view that the stoichiometry of the NMDA receptor is constant, in the sense that all receptors contain the same number of NR1 subunits, in the same position relative to the NR2A subunits, although clearly some fluctuation in assembly cannot be ruled out entirely. It would, of course, be possible for a receptor with two NR1 subunits to give rise to *more* than three distinguishable molecular species; the two NR1 positions in the oligomer are unlikely to be exactly equivalent in their properties, so it is possible that the conductance properties could depend on which of the two positions contained the wild-type subunit and which contained the mutant subunit. However, we observed only three species so this possibility does not affect our conclusions in the present case. The only plausible error that could lead to this conclusion being wrong would occur if other channel types were formed, but were not detected, when the mixture of wild-type and mutant cRNAs was injected, or if there were actually more than three channel types contributing to the channel openings that we have observed.

(*a*) *Do the three channel types described above each represent a single molecular species?*

In the case of wild-type channels, openings can be divided into three patterns. There are isolated openings to the '50 pS' level, isolated openings to the '40 pS' level, and there are openings that contain one or more direct transitions between these two levels. It is, therefore, not impossible that there are three sorts of wild-type channel. However, it is now generally accepted that all three sorts of opening arise from a single molecular species that can open to both conductance levels. The reasons for this are as follows (see, for example, Howe *et al.* 1991; Gibb & Colquhoun 1992; Stern *et al.* 1992): (i) there is close agreement (at least within any one experiment) between the conductances seen in isolated openings and those seen in openings that contain direct transitions; (ii) the relative frequencies of transitions between the various conductance levels is reasonably consistent from patch to patch (and is similar for native and recombinant receptors); and (iii) it would be surprising (assuming, as we have, that the NMDA receptor does possess a unique stoichiometry) if injection of a pair of wild-type subunits could give rise to three different types of channel. Similar arguments apply to the 'all-mutant channel' shown in figure 2, and to the 'mixed type' channel shown in figure 3. In both cases there is a close correspondence between the conductance levels seen in isolated openings and those seen in openings that contain direct transitions. The fraction of isolated openings of each conductance level, and the relative areas of the two components used to fit the amplitude distributions, did show substantial variability for the all-mutant (compare figure 4*c* and 4*d*) and for the mixed-type channels in these experiments, but the poor resolution, especially for the all-mutant channel, makes precise measurement of these quantities quite difficult, so the significance of this variability is not clear. Even if it were real, it is unlikely to have resulted from the presence of receptors with only one NR1 subunit because such receptors would be very unlikely to have exactly the same conductance properties as those with two NR1 subunits; Variations in relative positions of the NR1 and NR2A subunits would be a more likely cause. In any case, it would be very surprising indeed if wild-type, all-mutant and mixed-type channels all showed the same precise correspondence of conductance levels by chance. We are, therefore, confident that each represents a single molecular species.

(*b*) *Are there additional molecular species that have not been detected?*

Failure to detect additional channel type(s) could result from: (i) too few patches being inspected for a patch to be found that contained the additional type(s); (ii) the additional channel type(s) having a conductance that was too small to be resolved (or fail to express at all); or (iii) the additional channel type(s) having the same conductance characteristics as those that are observed. If, for example, there were actually three NR1 subunits, we would expect to see (at least)

two species in addition to the 'all wild-type' and the 'all mutant' molecules, namely those arising from molecules that contained two wild-type and one mutant subunit, and from molecules that contained one wild-type and two mutant subunits. It is very probable that such species would have conductance properties that were intermediate between the 'all wild-type' and the 'all mutant' molecules, so explanation (ii) is improbable. The evidence against explanation (iii) has already been discussed in the preceding paragraph.

A quantitative analysis of explanation (i) requires more knowledge than we possess, but an outline of the relevant considerations may be helpful. The relative efficiency with which each of the three cRNAs produces protein is the first unknown quantity. Secondly, the probability of each sort of subunit protein being incorporated into a functional receptor oligomer is unknown, (as is the probability of each sort of oligomer being inserted into the oocyte membrane). Finally, given that n_i molecules of the ith type are successfully incorporated into the membrane, the number of channel openings observed is unlikely to be directly proportional to n_i because there is no reason why the fixed glutamate concentration that is used to elicit openings should be at the same position on the concentration-response curve for each channel type.

Despite all of these uncertainties, the fact that patches have been seen with comparable numbers of openings for 'all wild-type' and 'all mutant' types, if it were interpreted as meaning that similar numbers of each type were expressed, might mean that there should be at least as many of the intermediate types present according to the prediction of a symmetrical binomial distribution. The following calculations refer to the case where the receptor actually contains $n = 3$ NR1 subunits; in this case, if the probabilities of insertion of a wild-type or a mutant subunit are equal ($p = 0.5$) then the binomial distribution predicts that the numbers of oligomers that contain $r = 0$, 1, 2, 3 wild-type subunits would be proportional to $P(0) = 0.125$, $P(1) = 0.375$, $P(2) = 0.375$ and $P(3) = 0.125$. If a membrane patch contains m channels altogether, then the number of channels of each of the four types in the patch would be distributed according to a multinomial distribution with the above probabilities. If, in addition, we assume that the number of channels per patch, m, is itself a random variable which follows a Poisson distribution with mean μ, then we can calculate the relevant probability. We are interested in the possibility that one or other of the two mixed receptor types ($r = 1$ or $r = 2$) might be absent from every patch, and so remain undetected. With the assumptions above we can calculate the probability of observing k patches in all of which the channel type with $r = 1$ is missing, or in all of which the channel type with $r = 2$ is missing. We have observed $k = 10$ patches, and if we suppose the average number of channels per patch was $\mu = 5$ this probability would be only 1.44×10^{-8}, and even if there were on average only $\mu = 3$ channels per patch this probability would be 2.6×10^{-5}. This argument, suggests that it is unlikely that an additional molecular species has been missed.

(*c*) *Other potential problems in the use of single-channel amplitudes*

The existence of conductance sublevels is clearly a potential problem for the method used here. However, in this study, the various conductance levels did not overlap and there was little ambiguity in identifying the number of channel types in each patch. A similar, but slightly more subtle, problem arises from the fact that there appears to be some genuine variability in the amplitudes of currents passing through NMDA receptor channels, both within one patch and between patches. The '50 pS' and '40 pS' levels are quite well defined in amplitude histograms (both here and in similar work, e.g. Gibb & Colquhoun 1992; Stern *et al.* 1992). However, in different experiments, under apparently identical conditions, the position of the '50 pS' level is somewhat variable (e.g. 47 to 55 pS), and even within one experiment the scatter of the measured channel amplitudes is substantially greater than it would be, for example, with adult rat muscle nicotinic receptors (see Colquhoun & Sigworth 1995, Fig. 14). Once again, this does not constitute a serious problem for the present work because of the good separation between the conductances for each species found here.

(*d*) *Implications of the results*

The finding of two NR1 subunits is consistent with the suggestion that there are two binding sites for glutamate and for glycine per NMDA receptor (Benveniste & Mayer 1991). The NR1 subunit is thought to contain a glycine binding site (Kuryatov *et al.* 1994; Wafford *et al.* 1994; Grimwood *et al.* 1995), and the fact that NR1 expresses (albeit very inefficiently) by itself in oocytes suggests that NR1 also contains a glutamate binding site. The NR1 subunit therefore appears play a role in the NMDA receptor which is somewhat analogous to the role of alpha subunits in nicotinic receptors.

5. CONCLUSION

In summary, the potential problems of interpretation all have satisfactory refutations, so we propose that the NMDA receptor contains two copies of the NR1 subunit. Similar experiments are in progress with the NR2A subunit. These experiments show a much more complex pattern of conductances which we cannot interpret fully at the moment; however the results do appear to be inconsistent with the idea that the NMDA receptor is a tetramer, but it could be a pentamer or hexamer.

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