

Single channel analysis of a novel NMDA channel from *Xenopus* oocytes expressing recombinant NR1a, NR2A and NR2D subunits

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1. Two types of subunit (an NR1 subunit and one of the NR2 subunits) are sufficient to form efficient NMDA receptors. In order to investigate whether functional receptors may contain more than one sort of NR2 subunit, NR1, NR2A and NR2D subunits were co-expressed in *Xenopus* oocytes.
2. Single channel recordings from the oocytes showed, as expected, channel openings that are characteristic of NR1/NR2A receptors (*ca* 50 and 40 pS conductances), and channels characteristic of NR1/NR2D receptors (*ca* 40 and 20 pS conductances). However, they also contained a novel channel that contained conductances of *ca* 30, 40 and 50 pS, with direct transitions between all three levels.
3. It is postulated that the novel channel contains NR1, NR2A and NR2D subunits. The implications of this for receptor stoichiometry are discussed.
4. The novel channel was intermediate between the NR1/NR2A and NR1/NR2D 'duplet' receptors in the length of the channel activations, and in the probability of being open during activation. Its glycine sensitivity was much higher than that of NR1/NR2A, and was comparable with that of the other 'duplet' receptors.

Glutamate receptors of the *N*-methyl-D-aspartate (NMDA) type are widely distributed in the central nervous system. In order to understand their function it is necessary to know which sorts of subunit make up native NMDA receptors, and how many of each are present. The answers to both of these questions remain uncertain. The need for both NR1 and NR2 subunits in a single receptor has now been clearly established (Monyer *et al.* 1992), and receptors that contain only two types of subunit can mimic closely the behaviour of most native receptors (Stern *et al.* 1992; Edmonds *et al.* 1995; Wyllie *et al.* 1996). However, the possibility that a single receptor contains more than one sort of NR2 subtype still requires further investigation (see Béhé *et al.* 1999; Hawkins *et al.* 1999; Dingledine *et al.* 1999).

Receptors are interesting only if they are functional, so it is essential to have functional as well as biochemical evidence for assembly of particular subunit combinations. Investigation of single channel currents is desirable because whole-cell characteristics are likely to be derived from a mixed receptor population (e.g. receptors that contain NR1 together with one of the NR2 subunits, and perhaps NR1 with two different NR2 subunits). The majority of work on 'triplet' receptors (those containing more than one NR2 type) has concentrated on the combination NR1/NR2A/NR2B,

but this combination is not ideal for a single channel study, because the conductances of channels formed from NR1/NR2A and NR1/NR2B subunits are very similar. However, NR1/NR2A and NR1/NR2D receptors have very different single channel characteristics, and hence NR2A and NR2D were chosen for this study.

Some evidence does exist for a native population of receptors containing both NR2A and NR2D. Both Sundström *et al.* (1997) and Dunah *et al.* (1998) have shown that NR2A and NR2D can be co-precipitated from certain tissues, for instance, adult human spinal cord, rat cortex and thalamus. This method does not, of course, show that such 'triplet' receptors are functional.

When NR1, NR2A and NR2D subunits are expressed simultaneously in a *Xenopus* oocyte, a new channel type is found in addition to the expected NR1/NR2A and NR1/NR2D types. This type does not yet seem to have been identified in recordings from native tissues, and the results presented here should be useful for comparison with future work in this area.

A preliminary report of some of our findings has appeared previously (Cheffings & Colquhoun, 1999).

METHODS

Oocyte expression procedures were as described in Béhé *et al.* (1995). Oocytes were obtained from *Xenopus laevis* that had been anaesthetised by immersion in a 0.2% solution of tricaine (3-aminobenzoic acid ethyl ester), decapitated and pithed. Oocytes were defolliculated and injected using a Drummond Nanoject with up to 50 nl of cRNA coding for either NR1a and NR2A, or NR1a, NR2A and NR2D subunits. The cRNA was synthesised as described previously (Bébé *et al.* 1995; Wyllie *et al.* 1996), using constructs provided by Dr R. Schoepfer (see Kuner & Schoepfer, 1996). For NR1a/NR2A injections, a roughly 1:1 ratio of the two cRNAs was used (as judged by ethidium bromide staining of a gel). For NR1a/NR2A/NR2D the amounts of NR2A and NR2D were chosen such that a similar whole-cell current (as measured by two-electrode voltage clamp at -70 mV in the presence of $10 \mu\text{M}$ glutamate and glycine) was obtained if they were injected separately with NR1a. This resulted in a ratio of roughly 1:1:200 for cRNAs for NR1a, NR2A and NR2D, respectively. Following injection, the oocytes were incubated at 19°C for 3 days and then stored at 4°C until needed. Oocytes were used 3–7 days after injection with cRNA.

Immediately before patch-clamp recordings were made, the vitelline membrane of each oocyte was removed in a hypertonic solution containing (mM): sodium methylsulphate 200, KCl 20, MgCl_2 1, Hepes 10 (pH 7.4, adjusted with KOH). The oocytes were transferred to a dish that was perfused continuously with external solution, which contained (mM): NaCl 125, KCl 3, NaH_2PO_4 1.25, Hepes 20, CaCl_2 0.85 (pH 7.4, adjusted with NaOH). Patch pipettes were made from thick-walled borosilicate glass (i.d. 0.86 mm, o.d. 1.5 mm, Clark Electromedical), and had their tips coated with Sylgard (Dow-Corning). After fire-polishing they had resistances of 10–20 M Ω . They were filled with an internal solution containing (mM): potassium gluconate 141, NaCl 2.5, Hepes 10, EGTA 11 (pH 7.4, adjusted with KOH). These solutions gave equilibrium potentials for K^+ and Cl^- ions of -100 mV. After formation of an outside-out patch, the patch was transferred to a separate compartment of the recording dish in order to prevent exposure of the oocyte to agonist-containing solution. Channel openings were elicited by 100 nM glutamate (in external solution), with either $20 \mu\text{M}$ (normal) or $0.5 \mu\text{M}$ (low) glycine. Single channel currents were recorded with an Axopatch-1D amplifier (Axon Instruments) and stored on digital audiotape (DAT) for subsequent analysis (Biologic DTR-1205).

For analysis, records were replayed from DAT tape, filtered at 2 kHz and digitised continuously at 20 kHz. Channel amplitude and duration of events were fitted using the SCAN program (see Colquhoun & Sigworth, 1995). This, and the other programs used for analysis are available at:

<http://www.ucl.ac.uk/Pharmacology/dc.html>

The shortest shut time that could confidently be identified was $45 \mu\text{s}$, so this resolution was imposed on the idealised results after fitting with SCAN. Distributions were fitted by the method of maximum likelihood using the EKDIST program (Colquhoun & Sigworth, 1995). In order to examine possible temporal changes in channel amplitudes, fitted amplitude stability plots were constructed of events with durations of at least $2T_r$, where T_r is the filter rise time. All of the channel amplitudes within a patch remained stable throughout the recording. Histograms of fitted amplitudes were also constructed from events with durations longer than $2T_r$. These were fitted with an appropriate number of Gaussian components. After fitting the amplitude histogram, critical amplitude (A_{crit}) values were calculated between each component so as to divide the amplitude range into a series of windows, each containing a single

component. The A_{crit} value was chosen such that the minimum total number of amplitudes was misclassified. These amplitude windows were used to classify and analyse all the transitions between amplitude components in a recording (Colquhoun & Sigworth, 1995). For this transition analysis, only openings with a duration of at least $2.5T_r$ were used. There is a certain amount of variability in the amplitudes of openings of NMDA receptors (stability plots show them to be far more variable than, for example, amplitudes of muscle nicotinic channels, despite the fact that amplitude histograms are rather consistent). This variability, together with noise, will result in some misclassifications being made.

Distributions of open periods were constructed and fitted with a mixture of three exponential components. Open periods were defined as a series of openings to one or more fitted amplitude levels which contained no resolvable (i.e. greater than $45 \mu\text{s}$) shuntings. Distributions of shut times were similarly constructed, and were fitted with five or six exponential components. For burst analysis, a critical shut time (t_{crit}) value was calculated between the two longest components of the shut time distribution. This value was chosen such that there were equal numbers of misclassified shut times, i.e. the number of events misclassified as being within a burst when in fact they were between, was equal to the number misclassified as being between when in fact they were within a burst (criterion of Magleby & Pallotta (1983) and Clapham & Neher (1984)). The data could then be divided into separate bursts that could be inspected visually for further analysis. By using the same amplitude windows that were calculated earlier, bursts could be classified according to which amplitude components they contained. An opening was included in this analysis only if it was at least $5T_r$ in duration, in order to reduce possible errors in amplitude classification.

RESULTS

Single channel amplitudes

Steady-state single channel currents from oocytes expressing NR1 with either NR2A or NR2D have been well characterised (Stern *et al.* 1992; Wyllie *et al.* 1996, 1998). Eight outside-out patches were examined from oocytes expressing all three subunit types; all patches contained similar amplitudes, but only four patches (52 980 transitions altogether) were suitable for the full analysis described below. These patches contained the amplitudes (mean values) expected for both NR1/NR2A (0.9, 3.9 and 5.2 pA at -100 mV) and NR1/NR2D (1.9 and 3.9 pA at -100 mV). It will be a convenient shorthand to refer to these as conductances of approximately 10, 40 and 50 pS for NR1/NR2A, and 20 and 40 pS for NR1/NR2D, when precision is not needed, though conductances were not determined from current–voltage relationships in this study. In our previous report (Stern *et al.* 1992), NR1/NR2A receptors were described as producing predominantly ‘40 pS’ and ‘50 pS’ levels. However, these values are not entirely consistent. In the present experiments the predominant levels were ‘40 pS’ and ‘50 pS’ as earlier, but a third amplitude of 0.9 pA (‘10 pS’) was observed more frequently than before. Re-analysis of earlier records showed this level to have been very rare, but in the present study this amplitude level was seen in all control NR1/NR2A recordings (see Fig. 1), and it was clearly connected to the two other levels. The ‘10 pS’ level now accounted for up to

5% of all openings in patches with NR1/NR2A. Possibly the combination of a change in the internal solution from Na⁺-based to K⁺-based, and the increased separation when recording at -100 mV instead of -60 mV, can account for the difference compared with earlier results, but it could also result from imperfection of the oocyte expression system. Some evidence is available to suggest that oocytes may not provide a perfect expression system for NMDA receptors. A *Xenopus* gene with homology to non-NMDA glutamate receptors has been shown to be present at low levels as a transcript in oocytes (Soloviev & Barnard, 1997). There is also evidence to suggest that the protein product of this gene may be able to form functional receptors in combination with NR1 homologues. It should be noted that the main level of NR1/NR2D superimposes completely on the larger sublevel of NR1/NR2A, and hence the combination of NR1/NR2A and NR1/NR2D in a single patch results in only four conductance levels ('10 pS', '20 pS', '40 pS' and '50 pS'), not five. In addition to these four amplitude levels, oocytes expressing all the three subunit types also contained a fifth, unexpected, amplitude level of around 3.1 pA, about '30 pS', as shown in Fig. 2.

Visually, it was clear that this novel 3.1 pA amplitude level was connected most often to the 3.9 pA level. However, there were a relatively large number of direct transitions between 3.1 and 5.2 pA (see Fig. 2 and Table 1), and many bursts contained all three levels: 3.1, 3.9 and 5.2 pA. This visual impression was supported by the transition analysis, performed for transitions between two amplitude levels as

described in the Methods (see Table 1). The table shows that the majority of the openings to 3.1 pA are connected to either the shut level or the 3.9 pA level, but a considerable number of them are connected to the 5.2 pA level.

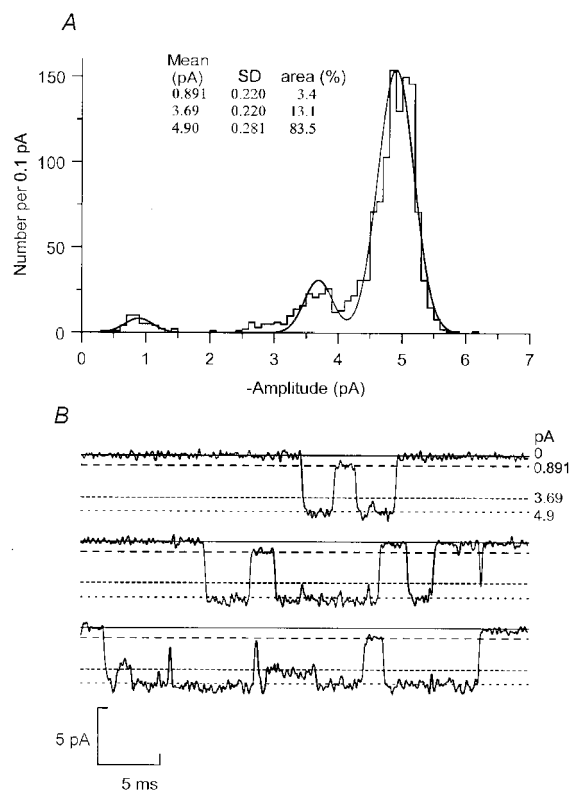
It can be seen in Table 1 that a small number of transitions were recorded between the 3.1 and 1.9 pA levels (30 pS and 20 pS in Table 1). According to our hypothesis such transitions would not be expected. However, it must be remembered that SCAN fits each current level solely on the basis of the data; there are no predefined levels to bias the fit. The amplitude stability plots (e.g. Figs 2 and 4) show that, as always with NMDA receptors, there was a substantial variability of amplitudes within each band. Therefore some misclassifications of amplitudes are inevitable. All such unexpected transitions were inspected (as were transitions between 3.1 and 5.2 pA), and, as shown in Fig. 3A and B, they corresponded to noisy or ambiguous levels in the record.

Transition analysis of NR1a/NR2A and NR1a/NR2D has already been published (Stern *et al.* 1992; Wyllie *et al.* 1996). In both cases the majority of transitions were between the shut state and an open state (87.6% for NR1a/NR2A, 90.3% for NR1a/NR2D). For NR1a/NR2A, 6.1% of transitions were 40–50 pS, and 6.2% were 50–40 pS. For NR1a/NR2D, 3.8% of transitions were 20–40 pS, and 5.9% were 40–20 pS.

It therefore seems apparent that a new channel type is present that has three open amplitude levels. Transitions are commonest between 3.1 and 3.9 pA or 3.9 and 5.2 pA,

Figure 1. Amplitude levels recorded from NR1a/NR2A channels

A, amplitude histogram of 1301 events recorded from an outside-out patch obtained from an oocyte expressing NR1a and NR2A subunits. Single channel currents were recorded at -100 mV in response to 100 nM glutamate + 20 μ M glycine. Only events with durations greater than $2T_r$ are included. The histogram is fitted with three Gaussian components with the values shown. *B*, representative traces from the same single channel recording, which includes examples of openings to the smallest sublevel, around 0.9 pA. Openings are downwards. Lines are drawn at the shut level (continuous line) and at the three amplitude levels fitted in the histogram. The smallest sublevel is clearly connected to the other two amplitude levels.



whilst there are less frequent transitions between 3.1 and 5.2 pA. In one patch the activity seemed to be mostly this novel channel type, as it did not contain NR1/NR2A (because it was recorded in a low glycine concentration; see below) and extremely few NR1/NR2D activations as judged by the rarity of '20 pS' openings (Fig. 4). This patch can be used to obtain the relative frequency of each of the three amplitude levels. The fitted amplitude histogram shows that, for openings longer than $2T_r$, most openings (51%) were to the 3.9 pA level, with 21% of openings to 3.1 pA and 28% of openings to 5.2 pA (shown with a fitted value of 5.3 pA in this case). Other patches, which contained more contamination from NR1/NR2A and/or NR1/NR2D, also clearly showed that the majority of openings were to the 3.9 pA level. It can also be seen from this 'pure' patch that the 0.9 pA level identified for NR1/NR2A is extremely rare or absent.

The conclusion of this analysis is that, in addition to NR1/NR2A and NR1/NR2D, there is a third channel type in these patches which possesses three connected amplitude levels of 3.1, 3.9 and 5.2 pA ('30–40–50 pS' channels). The two largest of these are identical with the two largest NR1/NR2A levels, thus complicating the analysis, but the '30 pS' level is not seen with either NR1/NR2A or NR1/NR2D.

Asymmetry of sublevel transitions

It has been observed that NR1/NR2D receptors show temporal asymmetry, transitions from the higher to the lower level being more common than transitions in the opposite direction (Wyllie *et al.* 1996). This means that the record is not at equilibrium, probably as a result of interaction between ion flux and gating (Lauser, 1983, 1985; Schneggenburger & Ascher, 1997). Similar asymmetry has been seen also in native channels in P1-P12 Purkinje

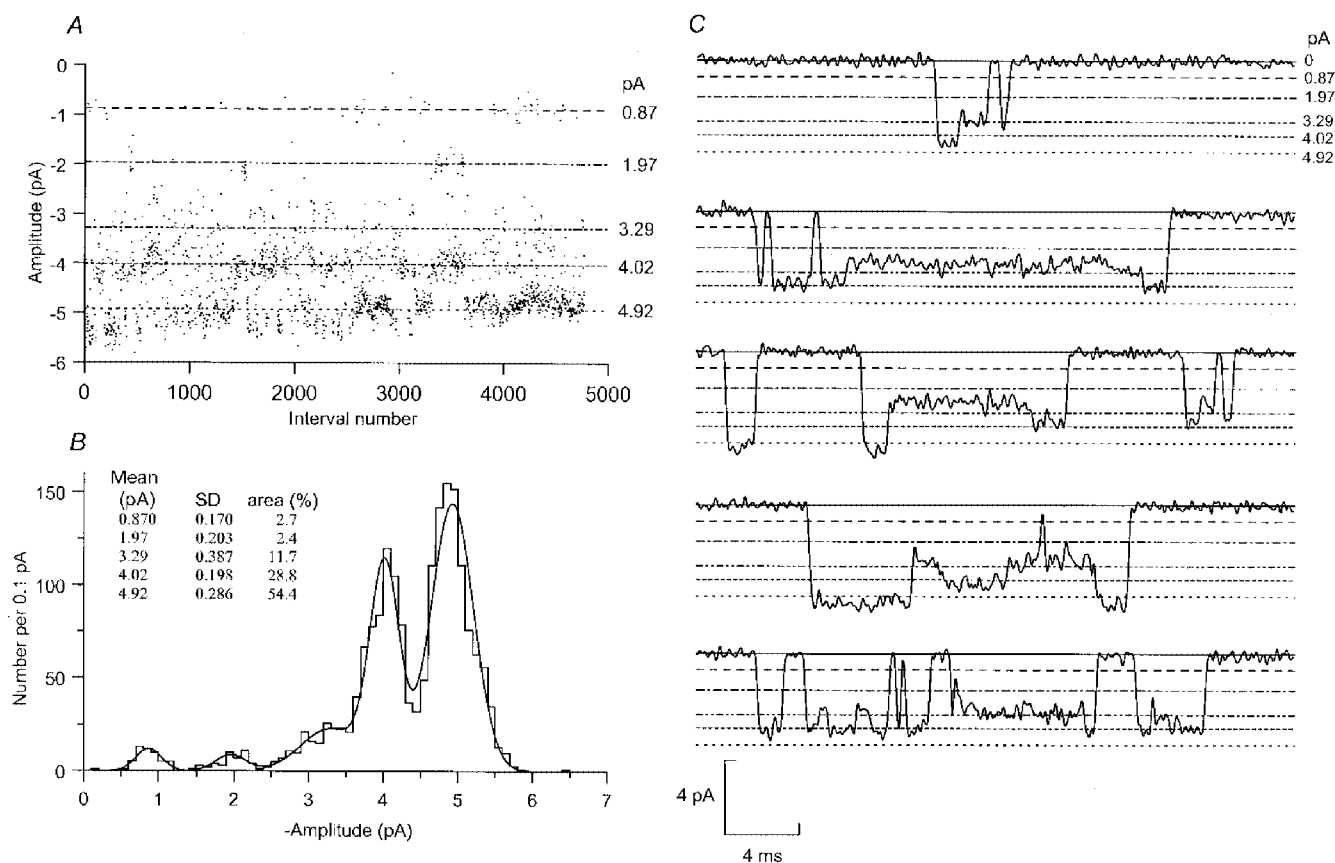


Figure 2. Amplitude levels recorded from oocytes containing NR1a, NR2A and NR2D subunits

A, amplitude stability plot of single channels recorded from an outside-out patch (No. 1 in tables) obtained from an oocyte expressing NR1a, NR2A and NR2D subunits. The recording was made at -100 mV in response to 100 nM glutamate + 20 μ M glycine. Only openings with duration greater than $2T_r$ are included. Lines are drawn at the amplitudes that were fitted in *B*. *B*, amplitude distribution of 1896 events from the same patch. Only openings with duration greater than $2T_r$ are included. The histogram is fitted with five Gaussian components with the values shown. *C*, representative traces from this same patch showing openings to, and connections of, the 3.29 pA amplitude level. Openings are downwards. Lines are drawn at the fitted values obtained in *B*, as for the stability plot in *A*, and also at the shut level (continuous line). Note that the inherent variability of this level has caused it to be smaller than the fitted value in traces 2 and 3. The 3.29 pA level is connected to both the 4.02 pA and the 4.92 pA levels.

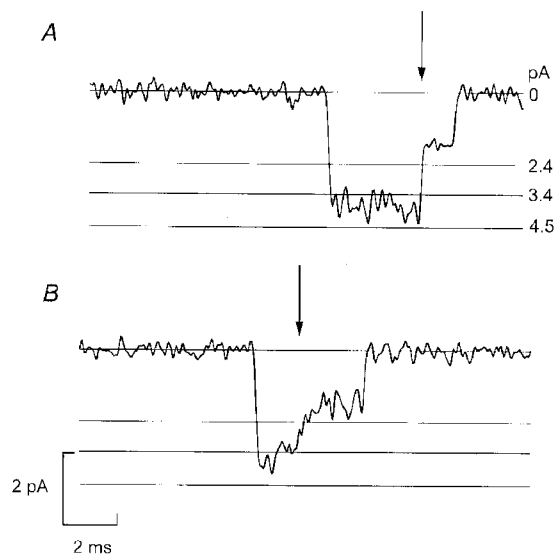
Table 1. Transition analysis

	Patch no.	Glycine conc.	Next level					
			Shut	10 pS	20 pS	30 pS	40 pS	50 pS
Transitions from 10 pS	1	Normal	0	NA	—	0	5.6	94.4
	2	Normal	0	NA	0	2.0	9.8	88.2
	2	Low	11.1	NA	3.7	11.1	33.3	40.7
	3	Normal	—	NA	—	—	—	—
	3	Low	—	NA	—	—	—	—
Transitions from 20 pS	4	Normal	—	NA	—	—	—	—
	1	Normal	—	—	NA	—	—	—
	2	Normal	71.8	0	NA	0	23.1	5.1
	2	Low	65.8	0	NA	13.9	16.5	3.8
	3	Normal	73.6	0	NA	3.2	20.2	3.0
Transitions from 30 pS	3	Low	77.5	0	NA	3.7	16.6	2.3
	4	Normal	—	—	NA	—	—	—
	1	Normal	74.8	0.1	—	NA	13.7	11.4
	2	Normal	64.4	0.5	0	NA	12.0	23.1
	2	Low	62.3	0.8	2.9	NA	26.0	7.9
Transitions from 40 pS	3	Normal	48.4	—	5.7	NA	42.2	3.7
	3	Low	50.5	—	7.3	NA	39.3	2.9
	4	Normal	60.0	—	—	NA	32.0	8.0
	1	Normal	80.8	0.5	—	10.7	NA	8.0
	2	Normal	83.4	0.9	2.4	4.0	NA	9.3
Transitions from 50 pS	2	Low	83.1	0.7	1.7	12.6	NA	1.8
	3	Normal	82.5	—	6.6	9.1	NA	1.9
	3	Low	78.6	—	7.7	11.6	NA	2.1
	4	Normal	75.5	—	—	17.2	NA	7.3
	1	Normal	88.0	1.8	—	5.6	4.6	NA
Transitions from 50 pS	2	Normal	86.3	3.9	0.3	4.8	4.7	NA
	2	Low	84.7	2.0	0.5	8.8	4.0	NA
	3	Normal	81.8	—	3.2	5.7	9.4	NA
	3	Low	75.3	—	4.2	8.4	12.1	NA
	4	Normal	82.4	—	—	7.8	9.8	NA

Values given are the number of the specified transitions as a percentage of all resolved transitions from that level. A dash is given when that conductance level was not observed in a patch.

Figure 3. Transitions from a patch (No. 3) with apparent connection between 20 and 30 pS

A, a normal transition (marked by arrow) between 40 pS and 20 pS levels. *B*, a noisy transition which is probably between the 40 pS and 20 pS levels. However, SCAN fitted an amplitude in the 30 pS range, so causing a connection to be recorded between 30 and 20 pS that is probably spurious. The lines mark the critical amplitudes used in analyses to divide the data into separate amplitude ranges.



cells (Momiya *et al.* 1996) which contain mRNA for both NR1 and NR2D (Akazawa *et al.* 1994). None of the other subunit pairs shows this asymmetry, so it is obvious to ask whether the novel NR1/NR2A/NR2D channel found here is asymmetric.

Transition analysis does not contain clear evidence for any asymmetry between the 30, 40 and 50 pS levels. Figure 5 shows a plot of amplitude against the next amplitude to which it is connected, for one of these patches, shown as two- (in *A*) and three-dimensional (in *B*) plots. It shows

clearly the asymmetry that is characteristic of the 'duplet' receptor, NR1/NR2D (40 to 20 pS transitions were more frequent than 20 to 40 pS), but no other asymmetries were detectable.

Separation of channel types by analysis of bursts

At sufficiently low agonist concentrations it should be possible to distinguish individual activations of a channel (for a definition of 'activation', see B  h   *et al.* 1999). During an individual activation only one channel type should be active. To investigate this, the data were divided into separate

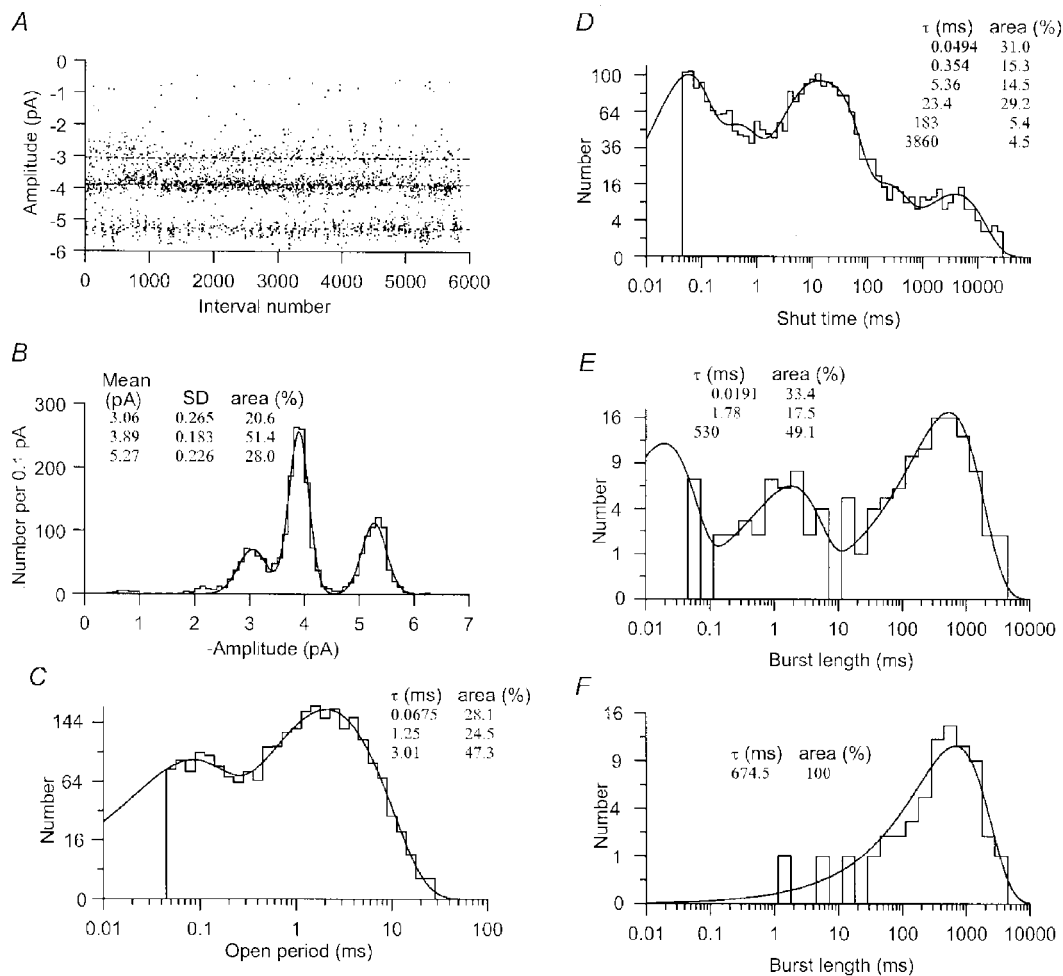


Figure 4. Analysis of a patch (recorded in low glycine) that contains mostly the putative NR1a/NR2A/NR2D channel

The recording was made from an outside-out patch (No. 2) held at -100 mV in response to 100 nM glutamate + 0.5 μ M glycine. *A*, amplitude stability plot showing that contamination by NR1a/NR2D is very low: three bursts of openings around 2 pA can be seen between intervals 4000 and 5000 which probably represent this contaminant. Lines are at the fitted values shown in the histogram in *B*. *B*, amplitude distribution of 2263 events from the same patch with durations greater than $2T_r$. The distribution has been fitted with three Gaussian components with the values shown. *C*, distribution of open periods to any amplitude level from the same patch. The distribution has been fitted with a mixture of three exponentials with the values shown. *D*, shut time distribution for the same patch fitted with a mixture of six exponentials with the values shown. *E*, burst length distribution for the same patch. The recording was divided into separate bursts using a t_{crit} of 450 ms, calculated from the open and shut time distributions. The burst length distribution has been fitted with a mixture of three exponentials with the values given. *F*, burst length distribution for the same patch and with the same t_{crit} but using the burst selection criteria detailed in the text. This distribution has been fitted with only one exponential component.

bursts as described in the Methods. Open and shut time distributions were not analysed in detail except to obtain a t_{crit} value from the shut time distribution, because they were obtained from a mixed channel population. In order to compare the burst length of the novel channel type with those of NR1/NR2A and NR1/NR2D, it was necessary to select bursts for analysis. The criteria that were used to select for bursts that originated from the novel channel were as follows.

- (1) Any burst that contained *only* poorly defined amplitudes (opening shorter than $5T_r$, or otherwise ill-defined) was rejected.
- (2) Any burst that contained an opening to the 1.9 pA level (as defined by an amplitude window around this level) was rejected.
- (3) Any burst that contained openings *only* to the 5.2 or 3.9 or 0.9 pA levels was rejected.

The second criterion will remove most NR1/NR2D bursts (though not those that contain only '40 pS' openings), or bursts that overlap a NR1/NR2D burst. There were a number of bursts which appeared to contain openings to both NR1/NR2D- and NR1/NR2A-like amplitudes (for instance see Fig. 6). These were interpreted not as being another intermediate-type channel, but rather to result from overlap of bursts that originate from two different channel types (a failure of burst separation that must inevitably occur occasionally). The third criterion should remove all NR1/NR2A bursts and the remaining NR1/NR2D bursts, but will also remove a subset of the novel

channel bursts, and will thus cause some bias in the final results, particularly by removing shorter bursts. Bias will also result from the application of the first criterion, which will remove all of the shortest bursts, of whatever type, from the distribution. Therefore, the distributions of total burst length do not contain all of the bursts for that channel type, and are strongly biased against the shortest components. The time constants of the longer burst length components should be relatively little affected by this bias, although the proportion of the distribution occupied by these components will be greatly distorted.

Bursts that were selected in this way had a burst length distribution the longest component of which was about 500 ms. This is longer than the slowest component of the burst length distribution for NR1/NR2A (201 ms, Wyllie *et al.* 1998), but much shorter than that reported for NR1/NR2D (5174 ms).

A useful comparison can be made between this burst selection analysis and the patch described earlier which was thought to contain the novel channel type but essentially no NR1/NR2A (because it was recorded in a low glycine concentration; see below) and extremely rare NR1/NR2D bursts. In this patch the distribution of the lengths of all bursts had time constants (τ) of 20 μ s, 1.8 ms and 530 ms (see Fig. 4). The largest component (with 49% of the area) is the slowest. These can be compared with those obtained if the burst selection criteria are applied to this same patch. After selection, only one component was visible, with a fitted value for τ of 670 ms. Although, as anticipated, the two faster components have been removed when the bursts are

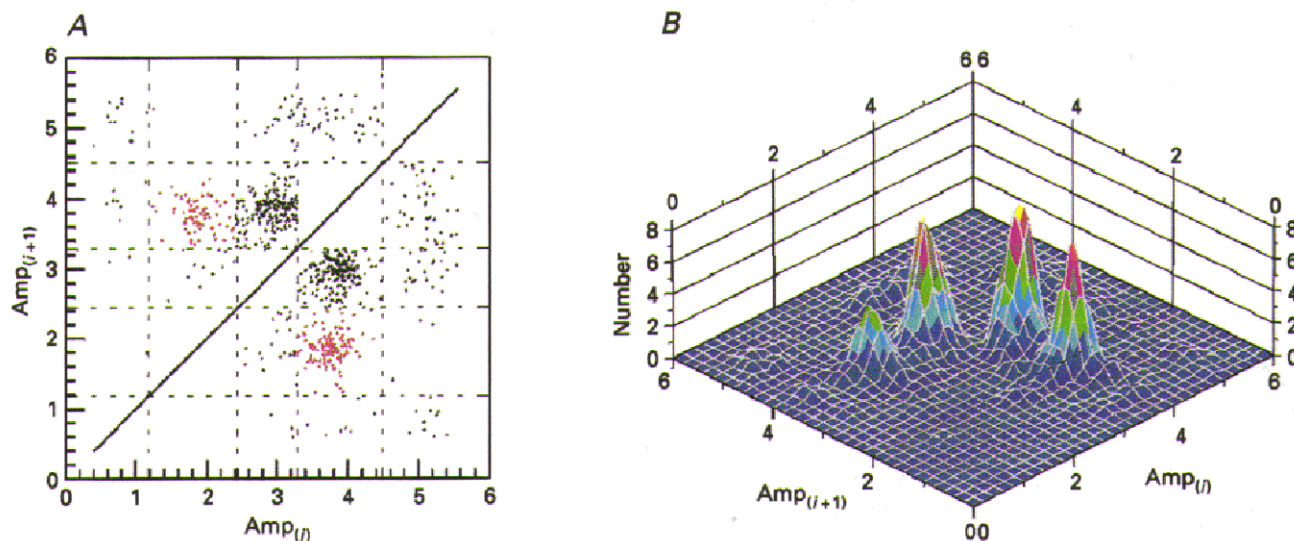


Figure 5. Transition plot for a single patch (No. 3) which contains the intermediate channel and NR1/NR2D

A, the amplitude of the i th channel opening (x axis) is plotted against the amplitude of the $(i+1)$ th opening that follows it (y axis): transitions to and from the shut state are omitted. The dashed lines show the amplitudes (A_{crit} ; see Methods) that divide the 10, 20, 30, 40 and 50 pS windows. The density of points is greater for 40 to 20 pS transitions than for 20 to 40 pS (both coloured red), as expected for NR1/NR2D receptors. This is more obvious in *B*, which shows the same data plotted as a three-dimensional histogram. The leftmost and rightmost peaks represent 20 to 40 pS and 40 to 20 pS transitions, respectively.

selected, the slowest component is relatively little affected. This comparison supports the earlier results, suggesting that the longest component is intermediate between NR1/NR2A and NR1/NR2D. The overall mean burst length in this patch was 705 ms when bursts are selected, and this is approximately halved to 374 ms when all the bursts from the patch are considered. Mean values from other patches are shown in Table 2. Judging by the result above, these are likely to be roughly twice the true mean burst length.

Fraction of time for which the channel is open during an activation

Another conspicuous difference between activations of NR1/NR2A and NR1/NR2D receptors is the probability that a channel is open within a burst (the 'burst P_{open} '). For NR1/NR2A this value is 0.35, giving a relatively 'tight-packed' appearance to the burst, whilst for NR1/NR2D it is only 0.04, so that the openings in the burst appear very sparse (Wyllie *et al.* 1998). These P_{open} values are calculated from the mean open time per burst divided by the mean burst length. The mean burst length was discussed above, and shown to be around 300–400 ms. The mean open time

per burst can, once again, be calculated only from selected bursts. This will, as previously, bias the results towards longer open times. However, it should be noted that both numerator and denominator are too large, so there will be some cancellation of errors in the estimate of burst P_{open} . Once again, comparison can be made with the patch believed to contain mostly the intermediate channel type; in this patch, the mean open time per burst for all bursts was 36 ms, whilst burst selection analysis on this patch gave a value of just over double at 82 ms. Further values are given in Table 2. This suggests that the P_{open} for the '30–40–50 pS' channel is around 0.15. Again this is intermediate between the values for NR1/NR2A and NR1/NR2D receptors, and is consistent with the visual impression of bursts sparser than NR1/NR2A, but more tightly packed than NR1/NR2D.

Glycine sensitivity of the novel channel type

The NR1/NR2A receptor requires a considerably higher glycine concentration than NR1/NR2D for full activation (Ikeda *et al.* 1992; Stern *et al.* 1992; Kutsuwada *et al.* 1992; Mori & Mishina, 1995). The glycine sensitivity of the

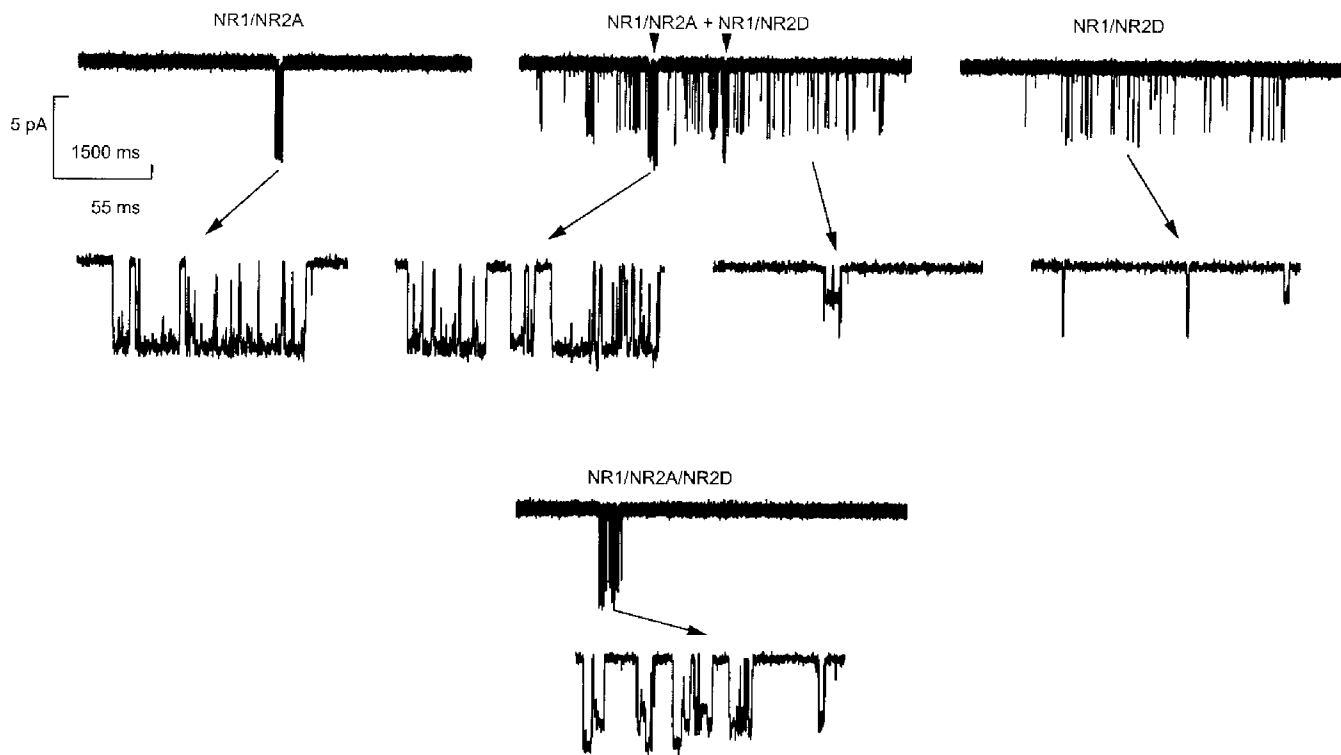


Figure 6. Comparison of four 'bursts' from the same patch showing the difference between 'pure' and 'overlapping' bursts

For each burst the complete burst is shown at the top with short expanded details shown below. Data from the same patch as Fig. 5 were divided into individual bursts using a t_{crit} of 580 ms. Whilst most bursts were easily classified as NR1/NR2A, NR1/NR2D or NR1/NR2A/NR2D, some appeared to contain amplitudes of both NR1/NR2A and NR1/NR2D; such a burst is shown in the centre of the top row. By comparison with the 'pure' NR1/NR2A and NR1/NR2D bursts on either side, it can be seen from the expanded details of this burst that it is most likely to be due to a single NR1/NR2D burst with two short NR1/NR2A bursts superimposed. The positions of the two NR1/NR2A bursts are marked with arrows. The 'overlapping' burst is completely different from the NR1/NR2A/NR2D burst shown beneath.

Table 2. Burst parameters for selected NR1a/NR2A/NR2D bursts

Patch no.	Glycine conc.	Mean burst length (ms)	Mean total open time per burst (ms)	Open probability within burst
1	Normal	194	17	0.09
2	Normal	756	58	0.08
2	Low	705	82	0.12
3	Normal	565	134	0.24
3	Low	812	146	0.18
Overall average	—	607	88	0.15
NR1a/NR2A	—	35.8	12.5	0.35
NR1a/NR2D	—	1602	65.8	0.04

Data for NR1a/NR2A and NR1a/NR2D are taken from Wyllie *et al.* (1998) and are shown for comparison.

intermediate channel type was also tested. Three outside-out patches were studied from oocytes expressing only NR1/NR2A receptors. In these patches, channel activations occurred in external solutions containing 20 μM glycine and 100 nM glutamate, but no activations were seen in solutions containing 0.5 μM glycine and 100 nM glutamate. It was this property of NR1/NR2A that was exploited in order to provide the patch (No. 2 in the tables) that contained mostly the novel channel type, because at low glycine concentration the recording was likely to have little contamination from NR1/NR2A (for which the response, in 0.5 μM glycine, should be less than 10% of that in 20 μM glycine). In patches from oocytes expressing NR1/NR2A, NR1/NR2D and putative NR1/NR2A/NR2D receptors, changing between the two glycine concentrations caused a marked reduction in the number of openings to the '50 pS' level (about 5 pA) (Fig. 7). This presumably results from a dramatic reduction in NR1/NR2A activations, but it may also be in part due to the intermediate channel making fewer openings to the '50 pS' level. However, what is immediately clear is that the intermediate channel still has frequent activations in low glycine concentrations, as does the NR1/NR2D channel. The distinction between a loss of NR1/NR2A activity and a reduction in openings to the '50 pS' level by the intermediate channel is one that is difficult to make. Only two patches were obtained in which recordings long enough for a detailed comparison were made at both glycine concentrations. In these patches, comparing the percentage of bursts containing particular amplitudes (see Table 3) showed a clear reduction at low glycine concentration in the number of bursts that contained only '50 pS' openings, or only '50 pS' and '40 pS' (but not the '30 pS') openings. In contrast the percentage of bursts containing all three levels ('30–40–50 pS') remained unchanged or increased. Therefore, whilst it does not seem to be the case that '30–40 pS' bursts increased at the expense of '30–40–50 pS' bursts, it still does not entirely rule out the possibility that within the '30–40–50 pS' bursts there may be fewer openings at the '50 pS' level. The

conclusion can only be that the novel '30–40–50 pS' channel does not clearly alter between the two glycine concentrations, and therefore, unlike NR1/NR2A, has quite high sensitivity to glycine.

DISCUSSION

The main conclusion of this work is that the novel '30–40–50 pS' channel is likely to be a 'triplet' receptor that contains NR1 together with both NR2A and NR2D subunits. This channel is intermediate in its properties between the

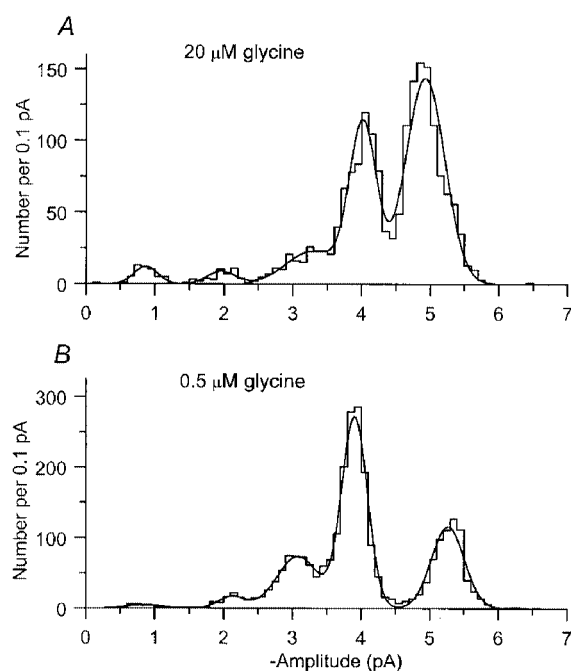


Figure 7. Comparison of the amplitude distribution at normal and low glycine concentrations

A, amplitude distribution in normal (20 μM) glycine. This figure is the same as Fig. 2B, but is reprinted here for ease of comparison. B, amplitude distribution for the same patch in low (0.5 μM) glycine.

Table 3. Conductance levels within a burst

Patch no.	Glycine conc.	Conductance levels (pS)															
		20	30	40	50	20	30	40	50	20	30	40	50	20	30	40	50
2	Normal	1	1	16	37	0	1	0	2	1	17	0	0	3	20	1	
2	Low	3	2	18	10	0	1	0	9	1	9	4	0	1	36	7	
3	Normal	4	1	23	17	0	10	0	9	1	4	6	0	2	16	7	
3	Low	0	0	18	3	0	23	3	5	0	0	15	0	3	13	20	

Each column represents the number of bursts that contain *only* the conductances that are listed at the top of the column, expressed as a percentage of the total number of bursts in the recording.

'duplet' receptors NR1/NR2A and NR1/NR2D receptors in the length of the channel activations and in the probability of being open during an activation. The length of the channel activations suggests that the response to a short concentration jump (which has not been measured directly) would probably be comparable to that seen with NR1/NR2B and NR1/NR2C receptors (Monyer *et al.* 1994; Vicini *et al.* 1998; Wyllie *et al.* 1998), a fact that could cause confusion in whole-cell studies.

The results raise an interesting question concerning the receptor stoichiometry. If the NR2D subunit is considered as a 'marker', in a manner analogous to the use of a mutated subunit by Béhé *et al.* (1995) or Premkumar & Auerbach (1997), then a single intermediate receptor type would imply that there were two NR2 subunits in the receptor: one NR2A and one NR2D. This would be in agreement with the findings of Premkumar & Auerbach (1997). However, it could be argued that the use of a different subunit constitutes a larger structural change than the use of a mutation within the same subunit, so, although mutated subunits might be able to assemble randomly, an NR1/NR2A/NR2D receptor might be able to assemble in only one way, in which case the number of intermediates might not reflect the number of subunits. In any case, to conclude that there are only two NR2 subunits requires certainty that there is really only one intermediate.

Clearly the question of whether the novel channel type described in this paper represents a single or multiple intermediates is an important one. The evidence suggests that in terms of amplitudes the '30–40–50 pS' channel cannot be split into multiple entities. However, this is not the only possibility. It is quite possible that all intermediate channel types have near-identical amplitude levels, but there could be two (or more) channel types differing, for instance, in burst length, percentage openings at each level, or glycine sensitivity. However, we cannot, with the data we have, make any such distinctions. The only conclusion possible at this stage is that our results are consistent with,

but do not demonstrate, the existence of two NR2 subunits in a receptor.

It is known that mRNA for NR1, NR2A and NR2D subunits can be found together, and co-precipitate, in adult human spinal cord (Sundström *et al.* 1997) and in cortex and thalamus of week-old and adult rats (Dunah *et al.* 1998). There is no direct evidence concerning whether or not the complexes detected by immunoprecipitation are functional or not. Data from *in situ* hybridisation are even more difficult to interpret, as assembly of subunits into a single receptor cannot be shown. However, the data of Akazawa *et al.* (1994) show that NR2A and NR2D are both present in the deep cerebellar nuclei. There do not seem to be any reports, up to now, of native channels that resemble our novel intermediate type. Ebralidze *et al.* (1996) found a range of conductance levels in wild-type mouse granule cells which express both NR2A and NR2C, but only one level when NR2C was knocked out. They suggest that this range of conductances may be due to NR1/NR2A/NR2C receptors, but they perform no detailed analysis of sublevels or connections between levels. It is possible that under physiological conditions, in which assembly is likely to be under a tighter control than in transfected cells or oocytes, no functional NR1/NR2A/NR2D channels are formed, and hence intermediates are not seen. This could also explain why studies of NMDA receptors that contain three different subunit types in oocytes have often suggested a more preferential assembly than co-precipitation from native tissues (see for instance Wafford *et al.* 1993, as compared with Sundström *et al.* 1997). However, it may well be that single channel studies, which would be needed to show unambiguously the triplet NR1/NR2A/NR2D receptor, have not yet been done on the right cell type, or have not been analysed in a way that reveals their characteristics.

If functional native NR1/NR2A/NR2D channels do exist, however, then they are likely to resemble the channel type described in this paper. Therefore this work provides a template against which native channels can be compared.

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