Ion Channels and Assembly

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Assembly of *N*-methyl-D-aspartate (NMDA) receptors

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Abstract

The *N*-methyl-D-aspartate receptor (NMDAR) requires both NR1 and NR2 subunits to form a functional ion channel. Despite the recent advances in our understanding of the contributions of these different subunits to both the function and pharmacology of the NMDAR, the precise subunit stoichiometry of the receptor and the regions of the subunits governing subunit interactions remain unclear. Since NR2 subunits are not transported to the cell surface unless they associate with NR1 subunits, cell-surface expression of NR2A can be used to monitor the association of the different subunits in cells transfected with N- and C-terminally truncated NR1 subunits. By combining measurements of cell-surface expression of NR2A with co-immunoprecipitation experiments, and by using Blue Native gel electrophoresis to determine the oligomerization status of the subunits, we have shown that regions of the N-terminus of NR1 are critical for subunit association, whereas the truncation of the C-terminus of NR1 before the last transmembrane region has no effect on the association of the subunits. Evidence from the Blue Native gels, sucrose-gradient centrifugation and size exclusion of soluble NR1 domains suggests that NR1 subunits alone can form stable dimers. Using a cell line, which can be induced to express the NMDAR following exposure to dexamethasone, we have shown that NMDARs can be expressed at the cell surface within 5 h of the recombinant gene induction, and that there appears to be a delay between the first appearance of the subunits and their stable association.

Introduction

The *N*-methyl-D-aspartate (NMDA) receptor (NMDAR) is one member of the glutamate-gated ion-channel family. The NMDAR has an important role in neuronal development, synaptic plasticity and several types of neurological disorders [1,2]. Unlike other members of the glutamate receptor family the NMDAR requires both NR1 and NR2 subunits to form a functional ion channel. The NR1 subunit is encoded by a single gene, which undergoes extensive splicing to generate eight different splice variants that differ in regional distribution and functional properties. The NR2 subunit class consists of four different subtypes, NR2A–NR2D, encoded by four separate but closely related genes (reviewed in [1]). A number of studies of mammalian cell lines, either permanently or transiently transfected with NR1 alone, have indicated that the NR1 subunit does not form glycine–glutamate-responsive channels and requires the presence of NR2 to do so [3–5]. Other studies have shown that the NR1 and NR2 subunits contribute differently to the binding sites of a functional NMDAR. The NR1 subunit forms the glycine-binding site [6,7] and the NR2 subunit provides part of the glutamate-binding site [8,9]. Thus different combinations of the two subunits must co-assemble to form functionally distinct NMDARs.

Despite the increase in our understanding of the molecular determinants underpinning the agonist binding, desensitization and modulator binding of the different NMDARs we still do not know the precise stoichiometry of the receptor, the regions of the subunits which are important for receptor assembly and the specificity of the subunit interaction, nor do we know how long it takes to make a functional NMDAR. These last two questions are addressed in this paper.

Time course of NMDAR assembly

Relatively few studies have investigated the time taken for ion-channel receptors to assemble. The best studied of these, the acetylcholine receptor, shows a fast association of the

Key words: ion channel, ion channel assembly, glutamate receptor, *N*-methyl-D-aspartate receptor (NMDA receptor).

Abbreviations used: LIVBP, leucine, isoleucine, valine binding protein; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; BN-PAGE, Blue Native gel electrophoresis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid.

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Figure 1 | Summary of NMDAR-induction experiments

The L(tk-) cells were induced to express the NMDAR by adding dexamethasone (arrow) and the production of the subunits and receptor monitored. The hours following induction are indicated by the horizontal boxes and the brackets indicate the range of time over which the different phases of subunit synthesis, association and oligomer formation were first detected. The upper shaded triangle shows the first detection of ion channels by electrophysiology and their increase in conductance with time, while the lower does the same for the appearance of oligomeric receptors as detected by BN-PAGE.

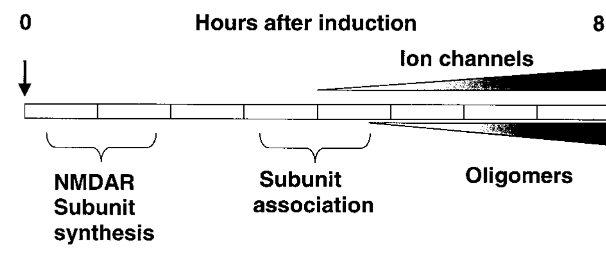
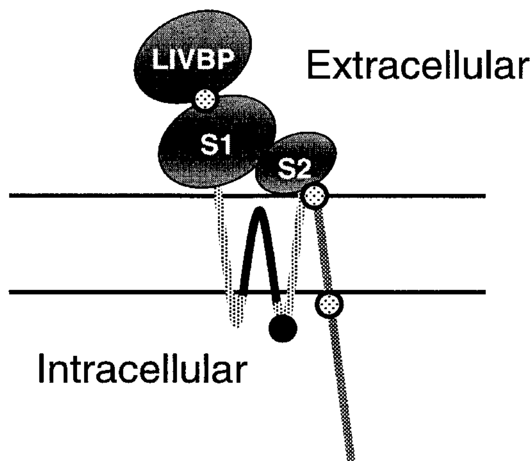


Figure 2 | Schematic of the topology of a glutamate-receptor subunit

The extracellular N-terminus is composed of two domains, the LIVBP and S1 domains. The former is important for the association of the subunits making up the functional receptor (see text) and the latter together with the non-contiguous S2 domain forms the agonist-binding pocket, in the case of the NR1 subunit the glycine-binding site. The solid black line indicates the recurrent loop that forms the ion channel in the intact receptor. The spotted circles show the limits of the N- and C-terminal truncations used in the study. The solid circle shows the most extreme truncation from the C-terminus, which removed all of the C-terminus up to the end of the region forming the ion channel. This construct did not give rise to surface expression of NR2A, suggesting that the N-terminus of NR1 alone does not associate with NR2A.



subunits, followed by a slow maturation phase in which the subunits are organized into the functional pentameric receptor. Thus while some subunit associations occur within minutes, there is a delay of up to 90 min before the functional receptor is formed [10,11]. In order to study the assembly process a robust and consistent expression system for the receptor is required. The availability of a stable L(tk-) cell line, which can be induced to express human heteromeric NR1a/NR2A receptors, provides such an expression system [12]. Following induction of the NR1a/NR2A-II stable cell line the synthesis of the NR1a and NR2A subunits can be monitored by immunoblotting, their association by co-immunoprecipitation and their oligomerization by Blue Native gel electrophoresis (BN-PAGE) or electrophysiology. Using this approach we have found that the synthesis of both subunits can be detected within 2 h of their induction by immunoblotting. Oligmerization of the two subunits can be detected by both BN-PAGE and electrophysiology within 5–6 h of induction, although the strength of both signals rises rapidly between 5 and 8 h. Surprisingly the co-immunoprecipitation data show that stable subunit association can only be detected 4–5 h after induction, long after the subunits are detected by immunoprecipitation and these data are summarized in Figure 1. Thus there appears to be a delay of over 1 h between the appearance of the subunits and their stable association.

The NR1 subunit forms the glycine-binding site, and has to associate with the NR2A subunit to make a glutamate-binding site. The subunits have then to assemble into a functional channel that allows the open channel blocker MK801 to bind. Radioligand-binding techniques can therefore be used to monitor the synthesis and assembly of the NMDAR. Using this approach we have found that within 8 h distinct glycine-binding sites can be detected, but glutamate-binding sites cannot be detected for a further 2 h, and coincide with the detection of MK801 binding. The delay in detection of the binding sites, compared with that of the subunits by immunoblotting, most probably reflects the different sensitivities of the two methods in detecting the subunits. However, it is striking that both methods suggest that the appearance of association of NR1a/NR2A compared with that of the folded NR1 subunit is delayed by 1 h or more. Therefore there appears to be a prolonged period after the synthesis of the two subunits before they associate and start to form oligomers and functional ion channels. This period must be made up partly of the time taken for the subunits to fold, and associate, as well as the time taken for their transport to the cell surface. The fact that there is a delay between the appearance of the glycine-binding site and the appearance of the glutamate-binding site suggests that folded NR1 subunits are present before the formation of the glutamate-binding site, by associating with NR2. Together the data suggest that the formation of functional NMDARs is a slow, ordered and complex process and that the correct folding of both NR1 and NR2 may be necessary for their association.

Subunit determinants important for NMDA assembly

The topography of a glutamate receptor subunit is thought to look as illustrated in Figure 2, with the extracellular N-terminus composed of two domains, the LIVBP (leucine,

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isoleucine, valine binding protein) domain, which is homologous with bacterial periplasmic binding proteins, and the S1 domain, which co-operates with the S2 extracellular domain to form the ligand-binding site. Evidence for this comes from the fact that the expression of the S1 and S2 regions linked together leads to the formation of soluble binding sites for agonists [13] (in the case of the NR1 a glycine-binding site [14]), and from the crystal structures obtained from the soluble S1–S2-binding sites [15,16]. The LIVBP domain appears to have multiple functions; it can interact with extracellular proteins [17], in NR2 subunits it contains determinants important for NMDAR modulation and desensitization (see [18]) and in the non-NMDA glutamate receptors it contributes to subunit association [19–21]. There are three membrane-spanning regions whereas the pore-forming region forms a re-entrant loop similar to that found in K^+ channels and contains the residues that determine the electrophysiological properties of the channel [22–24]. The C-terminus is intracellular and contains regions important for trafficking and targeting of the receptor [25,26].

In order to determine which region is important for the assembly of NMDARs we prepared a range of N- and C-terminal truncations of NR1a and co-expressed these with NR2A. Since we have shown previously that NR2A is not expressed at the cell surface unless it is co-expressed with NR1 [27], cell-surface expression of the NR2A subunit can been used to monitor subunit association as can co-immunoprecipitation experiments. The formation of oligomeric complexes was monitored using a modification of the BN-PAGE system [28], a non-denaturing gel electrophoresis method, and by sucrose gradient sedimentation. Cell-surface expression of the different combinations was monitored using both cell-surface labelling and a cell-surface ELISA. Both methods gave the same results showing that if the LIVBP domain of the NR1 subunit was removed completely, then surface expression of NR2A was abolished. The C-terminus of the NR1 subunit could be removed, including the last transmembrane domain, and still there was surface expression of NR2A. The limits of the deletions of the constructs used in the study are shown in Figure 2 and a full analysis of the constructs and the results can be found in Meddows et al. [29].

Subunit association, as assessed by co-immunoprecipitation of NR2A with NR1a, also showed a similar pattern, with the LIVBP domain of NR1 being necessary for subunit association, and likewise the C-terminus of NR1 and the last transmembrane domain could be removed without affecting the co-immunoprecipitation of NR2A. Thus the region important for subunit association appeared to be the LIVBP domain, as in the other glutamate-receptor subunits. However, constructs containing only the LIVBP domain of NR1 did not give rise to NR2A surface expression, nor to co-immunoprecipitation of NR2A, suggesting that the N-terminal domain of NR1 alone was not sufficient for the association of the subunits, unlike the behaviour of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [20].

Studies of subunit oligomerization using both sucrose gradients and BN-PAGE showed that the full NR1a subunit could form dimers when expressed alone, whereas the NR2A subunit seemed not to fold when expressed alone. Only when the two subunits were co-expressed did they form a 15 S complex on the sucrose gradients and an 880-kDa complex on the BN-PAGE gels [29]. One striking finding from these experiments was that when the NR1 subunit was truncated just before the last transmembrane domain, this construct was capable of supporting cell-surface expression of NR2A, its association with NR1a and the formation of an oligomeric complex with NR1a. However, this truncated form of NR1a was not able to form functional receptors, suggesting that the last transmembrane domain of NR1a is essential for channel formation, but does not play a role in subunit association and oligomerization.

In order to examine the role of the N-terminus of the NR1 subunit in its self-assembly, constructs containing either the LIVBP domain and the S2 region of NR1, or only the S1 and S2 regions of NR1, linked by a flexible peptide sequence, were produced. These were expressed in HEK-293 cells and the medium was found to contain the secreted proteins, as has been found by others [14,30]. Analysis of the secreted products by size-exclusion chromatography showed that the LIVBP-S2 form was secreted as a dimer into the medium whereas the S1–S2 form, although secreted, was aggregated with some monomeric protein. Thus the S1 region of NR1 is necessary for its dimerization. Co-expression of the former, but not the latter, with full-length NR2A subunits resulted in the low-level expression of NR2A at the cell surface. This confirmed our previous finding that the LIVBP region of NR1a is necessary for its interaction with NR2A.

Discussion

These findings indicate that the LIVBP domain of the NMDAR must interact with other regions of the receptor to form stable subunit associations. It has been reported that the LIVBP region of other glutamate-gated ion channels is important, and possibly sufficient, for specific subunit interactions [20,21]. However, in a detailed study using chimaeric AMPA subunits, Ayalon and Stern-Bach [21] showed that the LIVBP region, while a major contributor to subunit association, needs other regions of the protein to form stable oligomers. This is in agreement with our data showing that the S1 region of NR1 is necessary but not sufficient for subunit association. Furthermore, our finding that the last transmembrane domain of NR1 is not necessary for subunit association or oligomerization, but is essential for the formation of a functional ion channel, is in good agreement with the study of Schorge and Colquhoun [31] in which tandem NMDAR subunits were analysed to show that this domain is critical for channel formation. This and other studies have also suggested that the NMDAR is a tetramer, although no direct proof of this is currently available. Our data from both the BN-PAGE gels and the sucrose gradients

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are consistent with a tetrameric NMDAR, but do not provide unequivocal evidence for it.

The fact that NR1 forms dimers on its own is relevant to the organization of the mature oligomeric receptor, which must be an ordered arrangement of the receptor subunits. A range of studies has suggested that both AMPA receptors and NMDARs could be made up of a dimer of dimers, with the different subunits first forming homo-dimers that then combine with each other to generate the final tetramer [21,31]. Clearly the demonstration that NR1a can self-dimerize would be consistent with this hypothesis. However, we have been unable to show dimerization of NR2A expressed alone and indeed the evidence to date suggests that this subunit may not fold correctly when so expressed [29]. In this context it is interesting to note that there are few reports of NR2A subunits being able to bind glutamate when expressed alone. The fact that there was a significant delay before we were able to immunoprecipitate NR2A with NR1 from cells expressing both subunits means that the subunits may need some time to fold properly before they can associate, or that one of them may need to do this prior to the other associating with it. The fact that there is detectable glycine binding 1 h before a detectable glutamate-binding site would be consistent with the latter idea. Together the results presented here suggest that the formation of the NMDAR is a complex process requiring a significant maturation time for at least the NR1 if not the NR2A subunit to fold, and a complex interaction of both subunits to form a functional receptor, which is most probably a tetramer, containing two dimers of the different subunits.

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