Early events in the trafficking of \( N \)-methyl-\( \text{D} \)-aspartate (NMDA) receptors

R.J. Wenthold, N. Sans, S. Standley, K. Prybylowski and R.S. Petralia
Laboratory of Neurochemistry, NIDCD, NIH, Building 50, Room 4140, Bethesda, MD 20892, U.S.A.

Abstract
The \( N \)-methyl-\( \text{D} \)-aspartate (NMDA) receptor plays a central role at excitatory synapses where it has been implicated in multiple functions associated with synaptic plasticity. While this receptor has been intensely studied with respect to its physiology and pharmacology, its cell-biological properties, such as subunit assembly, post-translational processing and trafficking in neurons, are only beginning to be addressed. Critical to many of the functions of the NMDA receptor are the multiple proteins with which it interacts. While these interactions have been most thoroughly studied with respect to the receptor at the synapse, the same proteins may also interact with the receptor much earlier in its biosynthetic pathway and play important roles in receptor trafficking from the endoplasmic reticulum to the synapse.

Introduction
Recent studies show that glutamate receptors are regulated on at least two different levels. At the level of the cell, synthesis in the neuron is controlled by transcription and translation and also may be influenced through the regulation of subunit assembly and post-translational processing. An example of this level of regulation is the postnatal developmental change in the relative amounts of the \( N \)-methyl-\( \text{D} \)-aspartate (NMDA) receptor (NMDAR) subunits NR2A and NR2B [1]. During development there is a gradual decrease in NR2B and an increase in NR2A, leading to receptors with faster kinetics in older animals. Another example is the \( \alpha \)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPA) subunit, GluR2, which is downregulated by events such as ischaemia, generating AMPARs that are more calcium permeable [2]. In general, altering the rates of transcription and translation is effective in producing only slow changes in the number of receptors over a period of several hours to days. Glutamate receptors are also regulated at the level of the synapse. This is best characterized for the AMPAR, where it has been shown that the number of receptors at the synapse can be rapidly changed through endocytosis and exocytosis [3]. For example, stimulation of AMPARs or NMDARs leads to an internalization of AMPARs while generation of long-term potentiation of AMPARs or NMDARs leads to an internalization of AMPARs while generation of long-term potentiation

point of protein synthesis, the cell body. Thus changing the numbers of receptors by altering rates of transcription or translation would be ineffective in eliciting rapid changes in the number of receptors either throughout the neuron or in a subset of synapses. On the other hand, a local mechanism alone would not suffice in controlling receptors during periods where their total numbers may change dramatically, such as during development.

Most of the attention has focused on receptor regulation at the level of the synapse. While local regulation is of more immediate importance to the function of the synapse and to synaptic plasticity, the cellular regulation of receptors is a critical determinant of the functional properties of a synaptic receptor since the synapse can use only the receptors that are available to it. An integral component of the global regulation is the post-translational processing, subunit assembly, and trafficking that follows subunit synthesis. In this review, we will address some of the key points on the assembly and trafficking of NMDARs early in their biosynthetic pathways, which ultimately influence synaptic transmission.

Subunit assembly
Functional NMDARs are made up of NR1 and NR2 subunits which form a tetrameric complex. There is a single NR1 subunit, of which there are eight splice variants, and four NR2 subunits, NR2A–D. Current evidence suggests that the functional receptor contains two NR1 subunits and two NR2 subunits, which assemble initially as homodimers [5,6]. In the receptor complex, the NR2 subunits can be either the same or different, and a single neuron can have multiple receptor complexes; for example, cortical neurons express NR1, NR2A and NR2B and have receptor complexes composed of NR1/NR2A, NR1/NR2B and NR1/NR2A/NR2B [7]. The receptor complex can also contain two different NR1 splice variants [8]. Since receptors have different functional properties depending on their subunit and splice-variant
Retention of NR1 splice variants in the ER

A retention signal, RRR, in the C1 cassette and an export signal, STVV, in the C2′ cassette, regulate trafficking of the NR1 subunit in the ER. Only the NR1-1 splice variant is retained in its unassembled form. Based on data from [12].

Figure 1 | Retention of NR1 splice variants in the ER

A retention signal, RRR, in the C1 cassette and an export signal, STVV, in the C2′ cassette, regulate trafficking of the NR1 subunit in the ER. Only the NR1-1 splice variant is retained in its unassembled form. Based on data from [12].

Availability of the NR2 subunit controls the number of functional receptors

NMDARs have metabolic half-lives of about 1 day in cultured cerebellar granule cells [16]. However, the major component of NR1 decays with a half-life of only 1–2 h. This rapidly degraded component was shown to be an unassembled subunit that is retained in the ER, which is consistent with the findings of McIlhinney et al. [10] showing ER retention of unassembled NR1 and NR2. Retained, unused subunits of complex proteins are often degraded in this time frame. These results, however, also point out that NR1 is synthesized in considerable excess (estimated to be about 10-fold) of NR2 [16].

The excess of NR1 is more clearly demonstrated by physiological studies on cultured granule cells that were transfected to increase the production of NR1 or NR2 [17]. Overexpression of the NR1 splice variants, NR1-1 or NR1-4, did not change the whole-cell NMDA currents indicating that there was no addition of functional NMDARs on the cell surface. Both splice variants of NR1 had similar effects. In contrast, overexpression of NR2A or NR2B increased the number of functional NMDARs on the cell surface. These results are consistent with the presence of a large pool of NR1 retained in the ER and awaiting assembly with NR2 subunits; addition of more NR1 subunits to this pool would be expected to have no influence on the total number of receptors made. The fact that the synthesis of additional NR2 subunits leads to more functional receptors suggests that the availability of NR2 subunits is the critical factor in the regulation of NMDAR production.

The number of NR2 subunits, however, does not affect the number of functional NMDARs present at the synapse. Although more functional receptors are produced by overexpression of either NR2A or NR2B, leading to an increased number of receptors on the cell surface, they are restricted from entering the synapse and are presumably located at extrasynaptic sites. Therefore, a separate mechanism is used to control the number of NMDARs that are present at the synapse. The number of NMDARs present at the synapse can be changed under various experimental conditions. Blocking of NMDARs in cultured hippocampal neurons using the antagonist APV (2-amino-5-phosphonovaleric acid) causes a dramatic shift to a more synaptic localization of NMDARs [18,19]. This requires activation of cAMP-dependent protein kinase (PKA), and activation of this kinase alone is sufficient to induce synaptic clustering. Protein kinase C activation induces a rapid dispersal of NMDARs from the synapse [20]. These results point to phosphorylation as one factor that influences the number of NMDARs. Block of activity with TTX (tetrodotoxin) increases both synaptic NMDARs and AMPARs [21]. Subunit-selective entry into the synapse may be regulated by activity [22]. Finally, in some cases, overexpression of receptor subunits may influence the number of synaptic receptors; a transgenic mouse that overexpresses NR2B has larger NMDA-mediated currents in the hippocampus [23] but not in the visual cortex [24]. Although not nearly as labile as AMPARs [25], NMDARs also are internalized in heterologous cells and neurons through clathrin-mediated endocytosis [26,27]. Internalization is attenuated through interaction with PSD-95 (post-synaptic density-95) [26], suggesting that the receptor–MAGUK (membrane-associated guanylate kinase) interaction may play a critical role, not only for clustering...

compositions, and perhaps different distributions, how these complexes are formed and how this process is regulated remain important questions. Some of the regions of the molecules important for assembly have been identified [5], but nothing is known about how the differential assembly of either NR2 subunits or NR1 splice variants is achieved. This process may be regulated, as it is for AMPARs [9], or may be dependent solely on subunit availability.

Neither NR1 nor NR2 subunits form functional receptors when expressed alone in heterologous cells. Early studies showed that when expressed alone, these subunits are retained in the endoplasmic reticulum (ER) [10]; ER retention is a common feature of the quality control mechanism for complex proteins that ensures that unassembled or otherwise defective proteins are not allowed to reach the cell surface [11]. Subsequent studies, however, showed that only the NR1-1 splice variant, which is the major splice variant found in brain, is ER-retained [12–14]. Two distinct motifs in the C-terminal domain control the ER retention of the NR1 subunit. The RRR motif in the C1 splice cassette is an ER retention signal, and STVV, the PDZ-interacting domain of the C2′ cassette, serves as an exit signal (Figure 1). The combination of these two signals leads to four different mechanisms that control the ER retention of the four NR1 C-terminal splice variants. It is not clear why this subunit requires such an elaborate mechanism since it is not thought to have a functional role in its unassembled state, but it highlights the complexity involved in the processing of the NMDAR. It should also be noted that the NR1 subunit can assemble with the NR3 subunit to produce a functional glycine receptor [15].

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the receptor at the synapse, but also for keeping it on the cell surface.

Establishing a large excess of NR1 subunits and limiting the number of NR2 subunits ensures that newly synthesized NR2 subunits will assemble with NR1 subunits to produce functional receptors. Such a mechanism would facilitate the production of receptor complexes that reflect the current synthesis of NR2 subunits, and may play a role in the developing neuron when NR2B is replaced by NR2A as the predominant subunit. This leads to a functional change and produces a receptor with faster kinetics and a tendency to be more synaptic than extrasynaptic [28,29].

### A role for MAGUKs in the early trafficking of glutamate receptors

The discovery that the distal C-terminus of the NR2 subunit interacts with PSD-95 [30], known at the time as a major component of the synaptic density, was the beginning of a very fruitful search for other proteins that interact with the C-terminus and led to a characterization of their roles in trafficking and clustering of glutamate receptors. PSD-95 and related proteins were initially viewed as molecules that function principally at the synapse as scaffolding molecules that interact with multiple other proteins to form and maintain synaptic structure and facilitate signal transduction. The current evidence suggests that synapse formation begins with an accumulation of presynaptic components followed by postsynaptic components [31–33]. The relationship between MAGUKs and glutamate receptors in synapse development is unclear. In studies on cultured neurons, MAGUKs are often present with either NMDARs or AMPARs at newly formed synapses [18,31,34]. This does not appear to be a requirement, however, since synapses with MAGUKs alone and with receptors alone are fairly common. Another question concerns which MAGUK is present at the developing synapse. Most studies, using either immunocytochemistry or transfection with a tagged construct, have focused on PSD-95. In the intact, developing hippocampus, however, PSD-95 is not abundant at the synapse, while SAP102 (synapse-associated protein 102) is the major MAGUK found at the synapse [35]. PSD-95 emerges later, concomitant with the appearance of NR2A, and eventually is the predominant MAGUK at the PSD. The discrepancy between the immunocytochemical results in vitro and in vivo may be explained by the fact that many of the antibodies used are not selective for PSD-95, but recognize other MAGUKs. The results of the studies using expression of a tagged PSD-95 in cultures do not show that PSD-95 is in fact present at developing synapses, but rather show that PSD-95 can be concentrated at synapses if it is expressed. While it is agreed that the MAGUKs play critical roles in the function of NMDARs, many key questions remain. Where does the PDZ-receptor interaction begin or end? Is it transient or static? What is the relationship between MAGUKs and NMDARs in both developing and mature receptors? How do the various MAGUKs differ in their roles at the synapse?

The site where the receptor–PDZ-protein interactions begin is not established, but there is compelling evidence that it may occur before the receptor reaches the synapse. The first evidence of this was the finding that an interaction of the PDZ-interacting domain of the C2′ cassette of NR1 splice variants was required for the subunit to exit the ER, as noted above [12–14]. Although the PDZ-interacting site of the receptor was involved, these studies did not prove that the interaction involved a PDZ protein, leaving open the possibility that another protein interacts with this site and is responsible for ER exit of the C2′ cassette. More direct evidence that a PDZ protein interacts in the ER comes from studies on the AMPAR subunit, GluR1, which associates with the MAGUK SAP97 through its C-terminus [36]. Immunoprecipitation analyses on intact hippocampus showed that SAP97 interacts with a GluR1–GluR2 complex with immature glycosylation states, indicating that the receptors had not passed through the medial Golgi apparatus [37]. SAP97 was less abundantly associated with AMPARs that were present on the plasma membrane. These findings show that SAP97 interacts early with GluR1 and may play less of a role in organizing the receptor at the synapse, thought initially to be its major function.

Evidence is also developing that NMDARs interact with MAGUKs early in the secretory pathway [38,39]. In particular, SAP102 interacts with the NR2 C-terminal tail in the ER and Golgi apparatus. This is consistent with the distribution of SAP102, which, in addition to being present at the synapse like PSD-95, is also distributed throughout the cytoplasm where it may interact with various organelles including vesicles containing proteins destined for the postsynaptic membrane. Future research needs to address the role of the MAGUKs and other PDZ proteins in the early processing and trafficking of NMDARs. This interaction may be less important to the correct trafficking of the receptor than it is to the PDZ protein itself. Linkage to the receptor early in its biosynthetic pathway may be an important step in the trafficking of the PDZ protein to its site of function, such as the PSD. A similar conclusion was supported from studies linking the trafficking of potassium channels and PSD-95 [40].

### References


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