Forum Minireview

Functional Proteins Involved in Regulation of Intracellular Ca^{2+} for Drug Development: Desensitization of *N*-Methyl-D-aspartate Receptor Channels

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Abstract. *N*-Methyl-D-aspartate (NMDA) receptors are a principal subtype of excitatory ligand-gated ion channels with crucial roles in a variety of physiological and pathological processes in the mammalian central nervous system (CNS). In contrast to synaptic non-NMDA receptors that rapidly internalize, synaptic NMDA receptors have been shown to be rather static. However, recent accumulating evidence gives rise to the possibility that NMDA receptors may also undergo internalization. In our studies, repeated but not sustained stimulation by an agonist indeed induced desensitization of NMDA-receptor channels, which may be due to a decrease in the number of NMDA receptors expressed on cellular surfaces by internalization in a manner similar to the clathrin-induced endocytosis. Desensitization of NMDA-receptor channels provides a clue for the elucidation of fundamental mechanisms underlying dynamic regulation of the number of NMDA receptors at synapses, which is undoubtedly critical for the maintenance of both integrity and functionality in the CNS.

Keywords: NMDA receptor, intracellular Ca^{2+} , internalization

Increase in intracellular free Ca^{2+} ions through NMDA-receptor channels

The *N*-methyl-D-aspartate (NMDA) receptor is a subtype of the ionotropic glutamate (Glu) receptors supposed to be preferentially expressed on neurons in the central nervous system (CNS). Activation of NMDA-receptor channels leads to the influx of Ca^{2+} ions across cell membranes and subsequent elevation of intracellular free Ca^{2+} concentrations in neurons (1–3). Increased intracellular free Ca^{2+} ions are responsible for a variety of physiological and pathological events in the brain. These include neuronal development (4), plasticity (5), and cell death (6). In immature neurons, moreover, activation of NMDA receptors is absolutely required for cellular survival rather than cell death seen in mature neurons (7).

Seven different subunits have been characterized for NMDA-receptor channels in rat brain to date, with

several homologues in humans. In native NMDA receptors, these subunits are orchestrated in various combinations toward heteromeric assemblies for functional receptor channels permeable to Ca^{2+} ions. The primary structures of those seven subunits are deduced from the cDNA sequence. The essential NR1 subunit arises from one gene with eight functional splice variants, whereas both NR2 and NR3 subunits are coded by four and two different genes, respectively. The predicted amino acid sequences are identified for mature peptides for the latter subunits in rats, including NR2A, NR2B, NR2C, NR2D, NR3A, and NR3B (8–10). Studies in which NR1 gene is knocked out by targeted disruption suggest the crucial requirement of this subunit for brain development and survival (11, 12). Members of the second gene family, NR2A to NR2D, do not encode functional receptors by themselves, but their products assemble with the NR1 subunit, resulting in enhanced responses to NMDA and modified channel properties (13). Although the NR1 subunit is abundantly expressed in virtually all regions of the brain, NR2A to NR2D genes are differentially expressed in developing and

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adult rat brains (14–16).

NMDA-receptor desensitization by repeated stimulation

Although α -amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA) receptors that are also a subtype of ionotropic Glu receptors have been shown to rapidly internalize, NMDA receptors are often considered 'estable' in the plasma membrane (17–19). When cortical neurons were continuously exposed to NMDA, in fact, the increase in intracellular free Ca^{2+} ions is sustained for at least 30 min at conservative levels (20). However, recent evidence has raised the possibility that NMDA receptors may undergo regulated traffic to and from the cell surface (13, 18, 19, 21, 22). Figure 1 shows our recent findings on the possible desensitization of NMDA-receptor channels in terms of an increase in intracellular free Ca^{2+} levels determined by fluorescence imaging with a confocal laser-scanning microscope. When cultured rat cortical neurons were briefly exposed to NMDA twice with an interval of 25 min, the second exposure to NMDA led to a less potent increase in intracellular free Ca^{2+} ions than that found after the first exposure in neurons cultured for 9 and 15 days in vitro (DIV), but not in those cultured for 3 DIV (Fig. 1, middle panel). When the interval was prolonged from 25 to 45 min, however, the second exposure was less effective in increasing intracellular free Ca^{2+} ions than

the first exposure even in immature neurons as seen in mature neurons (Fig. 1, right panel). When the interval was shortened from 25 to 5 min, by contrast, a similarly potent increase was seen in intracellular free Ca^{2+} ions even after the second exposure irrespective of the cell maturity (Fig. 1, left panel). These data are suggestive of the possible desensitization of NMDA-receptor channels permeable to Ca^{2+} ions, which would occur in a manner dependent on the time interval between stimuli as well as neuronal maturity. Similarly, the amplitude of a test pulse is markedly reduced to a half of the initial control pulse following a series of 10 applications of 1 mM Glu (21). The possibility that such desensitization may be due to a change in the number of functional receptor channels expressed on cell surfaces is feasible. In our preliminary experiments, in fact, the number of NMDA receptors is downregulated in plasma membranes 25 to 45 min after a brief exposure to NMDA in mature cultured rat cortical neurons (unpublished data).

Mechanisms underlying NMDA-receptor internalization

Clues to molecular mechanisms underlying the internalization of NMDA-receptor channels would come from the findings that the NR2B subunit contains an internalization signal, YEKL, within its C-terminal tail (18). This motif is a member of the well-characterized family of tyrosine-based internalization signals present in a number of integral membrane proteins and typically localized to the distal end of the C-terminal tail. The $\text{Yxx}\Phi$ (where Φ is a hydrophobic residue) serves as a recognition motif for the endocytotic adaptor protein AP-2, which links a cargo to clathrin, thereby promoting the delivery of the cargo to clathrin-coated vesicles (13). Accordingly, the internalization of an NR1/NR2B assembly is prevented by co-expression of a dominant-negative dynamin construct known to disrupt clathrin-dependent endocytosis (18). Moreover, deletion of the internalization motif significantly stabilizes the NR2B subunit expressed at the cell surface in HeLa cells (18).

The NR1/NR2A-receptor downregulation is prevented by an inclusion of the tyrosine kinase Src in the pipette or by application of tyrosine phosphatase inhibitors, suggesting the dependence on tyrosine dephosphorylation (21). The aforementioned identification of a tyrosine-based internalization signal in the C-terminus of NR2A (similar to that found in NR2B) argues in favor of the possible participation of tyrosine dephosphorylation in the downregulation of NMDA-receptor channels. This motif mediates binding of NMDA receptors to the AP-2 complex and is crucial for receptor downregulation (13).

Fig. 1. Possible desensitization of NMDA-receptor channels. Cortical neurons were cultured for 3, 9, or 15 days in vitro (DIV) and loaded with fluo-3 AM by incubation for 50 min at 37°C for subsequent determination of intracellular free Ca^{2+} levels by fluorescence imaging. Cells were exposed to 50 μM NMDA for 5 min in the absence of MgCl_2 during determination of fluorescence intensity, followed by washing and subsequent second exposure to 50 μM NMDA for 10 min with intervals of 5 to 45 min after washing. Values are the mean $\pm$ S.E.M. in 6 to 8 independent determinations.

Fig. 2. Hypothesized model depicting possible steps leading to desensitization of NMDA-receptor channels. The endogenous agonist Glu would associate with NMDA-receptor channels for intracellular signaling and then dissociate from receptors to lead to the initiation of an assembly with AP-2 for desensitization and internalization processes. The endocytotic protein AP-2 could bind to membranous NMDA receptors destined for internalization, followed by promotion of the formation of clathrin-coat and subsequent endocytosis of NMDA receptors to result in downregulation of functional receptor channels expressed in the plasma membrane.

Concluding remarks

The evidence that NMDA receptors are also internalized through clathrin-dependent endocytosis is accumulating in the literature. Interestingly, our findings give rise to an idea that the dissociation, but not association, of an agonist could prime the internalization of NMDA-receptor channels assembled with AP-2 linked to clathrin in a particular situation (Fig. 2). Mechanisms for regulated desensitization of NMDA receptors may be responsible for several forms of long-term depression in the brain. As neuronal cell death would involve mechanisms relevant to overstimulation of the influx of extracellular Ca^{2+} through NMDA-receptor channels, elucidation of desensitization processes of NMDA-receptor channels could give us a new point of view toward the therapy and treatment of a variety of neurodegenerative disorders in human beings.

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