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Activation of NMDA receptors thickens the postsynaptic density *via* proteolysis

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ABSTRACT

The postsynaptic density (PSD) is a protein complex that is critical for synaptic transmission. Ultrastructural changes in the PSD are therefore likely to modify synaptic functions. In this study, we investigated the ultrastructural changes in the PSD in the hippocampal CA1 stratum radiatum following neuronal excitation. Oxygen–glucose deprivation-induced PSD thickening in hippocampal slice cultures was blocked by the N-methyl-D-aspartate (NMDA) receptor antagonist MK801. To gain more insight into the mechanisms underlying NMDA receptor-mediated PSD thickening, we assessed the area, length, and thickness of the PSD after NMDA treatment. The PSDs thickened with just 2 min of NMDA receptor stimulation, and this treatment was considered sublethal. When N-acetyl-leucyl-leucyl-norleucinal, an inhibitor of calpain, cathepsins, and the proteasome, was applied, NMDA-induced PSD thickening was abolished. Furthermore, the calcium-induced calcium release inhibitor, ryanodine, reduced NMDA receptor-mediated PSD thickening. These results suggest that NMDA receptor activation induces PSD thickening by proteolysis through intracellular calcium increase, including that induced by calcium.

1. Introduction

Excitatory synapses have an electron-dense structure at the postsynaptic membrane called the postsynaptic density (PSD). Neuronal excitation induced by ischemia produces ultrastructural changes in and a reorganization of the molecular composition of the PSD (Hu et al., 1998; Kovalenko et al., 2006; Martone et al., 1999). Many different molecules involved in synaptic transmission, including the N-methyl-D-aspartate (NMDA) receptor and PSD-95, are highly enriched in the PSD (Valtschanoff and Weinberg, 2001). As a result, ultrastructural changes in the PSD might be accompanied by a change in multiple critical neuronal processes, including synaptic transmission, neuronal plasticity, and survival. However, little is known about the intracellular mechanisms underlying the ultrastructural changes in the PSD.

Activation of glutamate receptors evoked by application of glutamate or NMDA *in vitro* mimics PSD thickening in ischemic neurons *in vivo* and in *in vitro* models of brain ischemia (Dosemeci et al., 2001, 2002). Additionally, NMDA receptors mediate the cellular responses triggered by ischemic episodes (Arundine and Tymianski, 2004). These findings suggest that NMDA receptor activation mediates ischemia-induced ultrastructural changes in the PSD.

NMDA receptor activation regulates the dynamics of critical PSD components, including the accumulation of calcium calmodulin-dependent protein kinase II (CaMKII) (Dosemeci et al., 2002; Merrill et al., 2005). The translocation of proteins, such as CaMKII, to the PSD is also observed after transient cerebral ischemia (Hu et al., 1998), suggesting that the accumulation of these proteins in the PSD contributes to the thickening of this structure. In contrast, PSD-95 and serotonin receptor subtype 2A are decreased in PSD fractions from brains exposed to ischemia (Hu et al., 1998). Therefore, PSD thickening may be attributed to an increase in the total amount of protein in the PSD following neuronal activation.

Three-dimensional studies have revealed that the thickened PSD in the postischemic brain is less condensed and regular in

Abbreviations: PSD, postsynaptic density; NMDA, N-methyl-D-aspartic acid; OGD, oxygen–glucose deprivation; ALLN, N-acetyl-leucyl-leucyl-norleucinal; CICR, calcium-induced calcium release.

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shape compared with the control brain (Martone et al., 1999, 2000). *In vitro* treatment with calpain, a calcium-activated cysteine protease, reduces the density and widens purified PSD from the rat forebrain (Dosemeci and Reese, 1995). Moreover, spectrin and tubulin, which are enriched in the PSD, are proteolyzed (Dosemeci and Reese, 1995). *In vitro* models of ischemia have also shown that calpain activation is enhanced, resulting in spectrin degradation (Meloni et al., 2011). Calpain activation after ischemia also results in cleavage of the scaffolding protein PSD-95 (Gascón et al., 2008). The proteolytic degradation of cytoskeletal and scaffolding proteins by calpain may cause loosening of the PSD structure.

In another family of cysteine proteases, cathepsins cause a change in spine morphology *via* the proteolysis of myristoylated-alanine-rich C-kinase substrate and F-actin in hippocampal neurons exposed to NMDA (Graber et al., 2004). F-actin depolymerization by latrunculin A thickens the PSD of hippocampal neurons (unpublished data). Therefore, cathepsin-mediated loss of F-actin may be involved in PSD thickening following NMDA receptor activation.

In this study, we investigated if PSD thickening in hippocampal CA1 stratum radiatum in an *in vitro* model of ischemia is mediated by NMDA receptor activation. We also examined the effects of N-acetyl-leucyl-leucyl-norleucinal (ALLN)—an inhibitor of calpains—and cathepsins and the proteasome on the PSD structure after NMDA receptor activation to clarify the molecular mechanisms underlying PSD thickening.

2. Materials and methods

2.1. Organotypic hippocampal slice cultures

All experimental procedures and handling of animals were approved by the Animal Research Committee at the University of Hyogo, Hyogo, Japan. The number of animals used and their suffering were minimized in this study. Brains of deeply anesthetized Sprague Dawley rats (Japan SLC, Shizuoka, Japan) of either sex at postnatal day 7 were isolated, and 350-μm thick transverse slices were cut from the hippocampi using a McIlwain tissue chopper (Mickle Laboratory Engineering, Guildford, UK) in ice-cold Neurobasal Medium A (Life Technologies, Carlsbad, CA, USA). The slices were placed onto membrane units (0.4 μm; Millicell-CM, Millipore, Billerica, MA, USA) in 3.5-cm-diameter dishes containing 1.1 ml of culture medium (50% MEM, 25% HBSS, 25% horse serum, supplemented with 6.5 mg/ml glucose, 100 U/ml penicillin and 100 mg/ml streptomycin) and incubated at 37 °C for 7 days and at 34 °C for an additional 3–5 days in 5% CO_2. Culture medium was changed every 3–4 days.

2.2. Induction of oxygen–glucose deprivation (OGD)

The hippocampal slice cultures were placed in an oxygen-free chamber in deoxygenated 95% N_2/5% CO_2-saturated artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl (124), KCl (3), $NaHCO_3$ (26), $CaCl_2$ (2), $MgSO_4$ (1), KH_2PO_4 (1.25), and sucrose (10) for 60 min and then immediately fixed for TEM observation.

2.3. Treatment of hippocampal slice cultures

Organotypic hippocampal slices were pretreated with the NMDA receptor antagonist MK801 (10 μM), ALLN (10 μM), or the specific inhibitor of calcium-induced calcium release (CICR), ryanodin (10 μM), followed by OGD for 60 min or 50 μM NMDA for 2 min. Slices were also subjected to a co-treatment of the nNOS inhibitor L-NG-nitroarginine methyl ester (L-NAME) (10 μM) with either OGD for 60 min or 50 μM NMDA for 2 min.

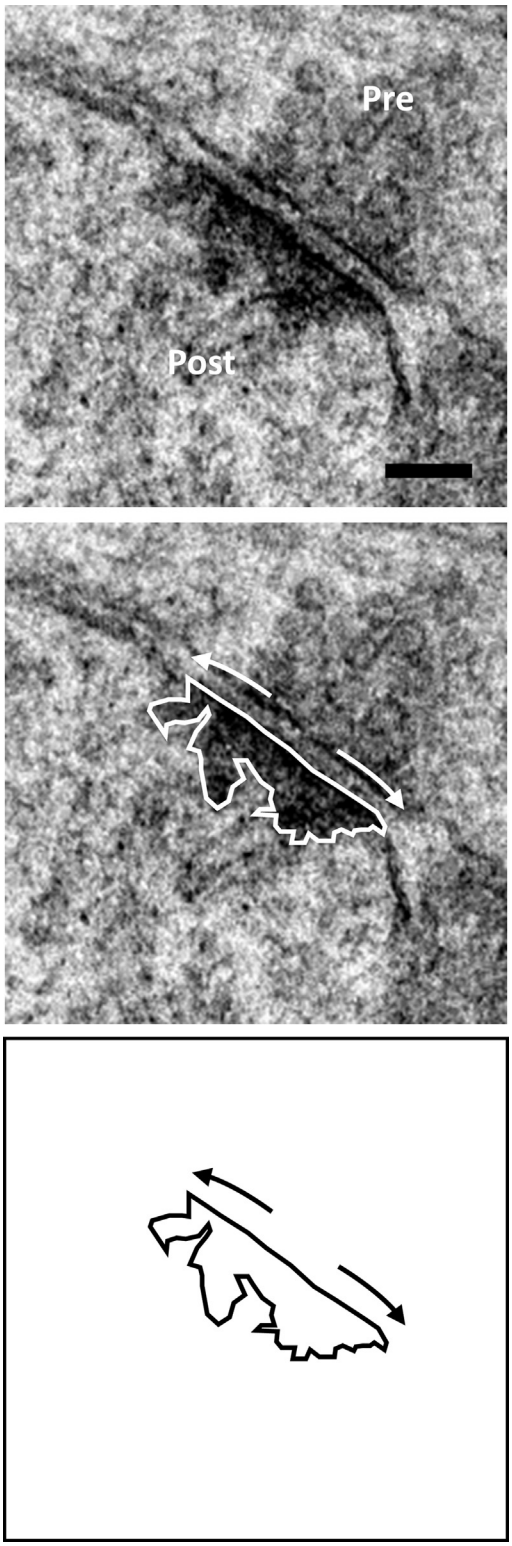

Fig. 1. Calculation of the thickness of the postsynaptic density (PSD) in the CA1 stratum radiatum of the hippocampus. The upper panel shows a representative electron micrograph of a synapse in the hippocampal CA1 region of slice cultures exposed to N-methyl-D-aspartate (NMDA) for 60 min. The PSDs are distinguishable from the cytoplasm, even if the thickened PSD of the hippocampal neurons exposed to OGD or NMDA is loose and irregular. The extracellular face of the postsynaptic membrane and the cytoplasmic face of the PSD was manually outlined to measure the area of the PSD as shown in the middle panel. The extracellular face of the PSD was separately outlined to measure the length of the PSD (between two arrows). The outline drawing of the PSD is shown in the lower panel. Scale bar = 100 nm. Pre, presynaptic specialization; post, postsynaptic specialization. The average thickness of the PSD was calculated by dividing the total area of the PSD by the length of the PSD.

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