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ULTRASTRUCTURAL QUANTIFICATION OF GLUTAMATE RECEPTORS AT EXCITATORY SYNAPSES IN HIPPOCAMPUS OF SYNAPSIN I+II DOUBLE KNOCK-OUT MICE

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Abstract—Previous findings, mainly in *in vitro* systems, have shown that the density of vesicles and the synaptic efficacy at excitatory synapses are reduced in the absence of synapsins, despite the fact that transgenic mice lacking synapsins develop an epileptic phenotype. Here we study glutamate receptors by quantitative immunoblotting and by quantitative electron microscopic postembedding immunocytochemistry in hippocampus of perfusion fixed control wild type and double knock-out mice lacking synapsins I and II. In wild type hippocampus the densities of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor subunits were higher (indicated for glutamate receptor subunit 1, highly significant for glutamate receptor subunits 2/3) in mossy fiber-to-cornu ammonis 3 pyramidal cell synapses than in the Schaffer collateral/commissural-to-cornu ammonis 1 pyramidal cell synapses, the two synapse categories that carry the main excitatory throughput of the hippocampus. The opposite was true for *N*-methyl-D-aspartate receptors. The difference in localization of glutamate receptor subunit 1 receptor subunits was increased in the double knock-out mice while there was no change in the overall expression of the glutamate receptors in hippocampus as shown by quantitative Western blotting. The increased level of glutamate receptor subunit 1 at the mossy fiber-to-cornu ammonis 3 pyramidal cell synapse may result in α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors with reduced proportions of glutamate receptor subunit 2, and hence increased Ca^{2+} influx, which could cause increased excitability despite of impaired synaptic function (cf. [Krestel HE, Shimshek DR, Jensen V, Nevian T, Kim J, Geng Y, Bast T, Depaulis A, Schonig K, Schwenk F, Bujard H, Hvalby O, Sprengel R, Seeburg PH (2004) A genetic switch for epilepsy in adult mice. J Neurosci 24:10568–10578]), possibly underlying the seizure proneness in the synapsin double knock-out mice. In addition, the tendency to increased predominance of *N*-methyl-D-aspartate receptors at the main type of excitatory synapse onto cornu ammonis 1 pyramidal cells might contribute to the seizure susceptibility of the synapsin deficient mice. The results showed no significant changes in the proportion of 'silent' Schaffer collateral/commissural synapses lacking α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors or in the synaptic membrane size, indicating that plasticity involving these parameters is not preferentially triggered due to lack of synapsins. **© 2005 Published by Elsevier Ltd on behalf of IBRO.**

Key words: GluR1, GluR2/3, NMDAR, postembedding immunogold, mossy fiber synapse, Schaffer collateral/commissural synapse.

Synapsins are a family of neuron-specific phosphoproteins that associate with synaptic vesicles (Greengard et al., 1993; Hilfiker et al., 1999, 2005; Dresbach et al., 2001). Previous studies suggest that synapsins have several different functions in the presynaptic terminals, including regulation of the recruitment of synaptic vesicles from a reserve pool and regulation of the fusion of synaptic vesicles with the plasma membrane (Hilfiker et al., 1999, 2005). Synapsins are also associated with the regulation of neuronal development, maintenance of mature synapses and synaptogenesis (Ferreira and Rapoport, 2002). Morphological and physiological evidence indicates that synapsins are involved in maintaining a reserve pool of synaptic vesicles (Li et al., 1995; Pieribone et al., 1995; Rosahl et al., 1995; Takei et al., 1995; Bloom et al., 2003). Double knock-out mice, lacking synapsins I and II (DKO mice) are epileptic, which appears to contrast with the impairment of excitatory synaptic function observed on repetitive stimulation. We therefore hypothesized that changes in the densities of glutamate receptors could explain the seizure activity. The possible influence of synapsins on the distribution of synaptic receptors does not appear to have been explored. Here we quantify the distributions of major types of glutamate receptors (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, subunits glutamate receptor subunit 1 (GluR1) and glutamate receptor subunits 2 and/or 3 (GluR2/3), and *N*-methyl-D-aspartate (NMDA) receptors, subunits NR1 plus NR2A/B) in the two main categories of synapses in the hippocampal throughput pathway, i.e. the mossy fiber (mf)-to-cornu ammonis (CA) 3/4 pyramidal cell synapses and CA3 pyramidal cell axon-to-CA1 pyramidal cell (scc, Schaffer collateral/commissural fiber(s) [associational and commissural axon branches from cornu ammonis 3 pyramidal cells]) synapses, in control wild type (WT) and DKO mice.

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Abbreviations: AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CA1–4, cornu ammonis 1–4 [hippocampal subfields]; DKO, double knock-out; GluR1, glutamate receptor subunit 1; GluR2/3, glutamate receptor subunits 2 and/or 3; HSA, human serum albumin; mf, mossy fiber(s); NaPi, sodium inorganic phosphate; NMDA, *N*-methyl-D-aspartate; NMDAR, *N*-methyl-D-aspartate receptor(s) (e.g. NR1, NR2A/B); PSD, postsynaptic density; scc, Schaffer collateral/commissural fiber(s) [associational and commissural axon branches from cornu ammonis 3 pyramidal cells]; TBST, Tris-buffered saline with Triton X-100; WT, wild type.

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These projection systems were characterized by Theodor W. Blackstad and his students in the early days of modern neuroanatomy (Blackstad, 1956; Blackstad and Kjaerheim, 1961; Hjorth-Simonsen, 1973). Beside his devotion to electron microscopy, which he pioneered, two of Blackstad's overriding scientific ideas were that morphology should be analyzed in quantitative terms and that the chemical composition of the tissue should be mapped—for which he used the term "chemoarchitectonics." The theme of the present paper is therefore directly in line with Theodor Blackstad's visions. We dedicate it to his memory.

EXPERIMENTAL PROCEDURES

Animals

Mice devoid of synapsin I and II (synapsin DKO) were obtained by homologous recombination as previously described (Chin et al., 1995; Ferreira et al., 1998). Animals were kept under conditions of constant temperature (22±2 °C) and relative humidity (55±5%), a 12-h light/dark cycle and free access to food and water, and used at postnatal day 28–60. Animals were treated according to the Norwegian Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC). Efforts were made to minimize animal suffering and to reduce the number of animals used.

Assay for biochemical quantitation of glutamate receptors

WT and synapsin DKO mice were killed by cervical dislocation and the hippocampus rapidly removed and stored in ice-cold 0.32 M sucrose. The hippocampus was homogenized in 0.32 M sucrose (5% w/v) in a glass-Teflon homogenizer at 450 r.p.m. and SDS added to (final concentration 1%).

After protein content quantification (BCA assay, Pierce, Rockford, IL, USA), samples in 1% SDS were added to sample buffer (final concentration 2% SDS, 10% glycerol, 50 mM Tris/HCl (pH 6.8), 0.25% Bromophenol Blue, 0.1 M dithiothreitol) and boiled for 2 min. Equal amounts of total proteins (10 μg/lane) from synapsin DKO and WT mice were subjected to SDS–polyacrylamide gel electrophoresis on 7.5 or 10% polyacrylamide gels. The separated proteins were electrophoretically transferred overnight (Towbin et al., 1979) to nitrocellulose membranes (0.2 μm pore size, BioRad, Richmond, CA, USA) and general transfer efficiency was evaluated by staining the membranes with 0.2% Ponceau S (Salinovich and Montelaro, 1986). Individual proteins were quantitated by immunoblotting with different primary antibodies (GluR1) (dilution: 0.01 μg/ml) (gift from R. J. Wenthold), GluR2/3 (dilution: 0.02 μg/ml) (Chemicon, Temecula, CA, USA), *N*-methyl-D-aspartate receptor (NMDAR) 1 (dilution: 0.02 μg/ml)(gift from R. J. Wenthold), NMDAR2A/B (dilution: 0.05 μg/ml) (Chemicon), employing enhanced chemiluminescence (ECL) Plus reagent (Amersham, Buckinghamshire, UK) for detection. The signals were analyzed using either an Autochemi system (UVP, Cambridge, UK) or using Hyperfilm MP (Amersham), scanned in a desktop scanner (Hewlett Packard Scan Jet 3c) at 600 d.p.i. and quantified densitometrically with Adobe Photoshop (v 7.0). The results were statistically evaluated by Student's *t*-test.

Tissue preparation and postembedding immunogold labeling

Mice were deeply anesthetized by an i.p. injection of Equithesin (0.3 ml per 100 g b.w.) and subjected to transcardiac perfusion with a mixture of glutaraldehyde (0.1%) and formaldehyde (4%) in 0.1 M sodium phosphate buffer pH 7.4 (NaPi).

Freeze substitution and further processing followed principally previous work (Bergersen et al., 2003) as detailed here. Rectangular specimens (typically 0.5 mm×0.5 mm×1 mm) from hippocampus were cryoprotected by immersion in graded concentrations of glycerol 10%, 20% and 30% in NaPi 0.1 M pH 7.4 for 30 min at each step and then overnight at 4 °C in 30% glycerol in 0.1 M NaPi. Samples were then plunged into liquid propane cooled at −190 °C by liquid nitrogen in a Universal Cryofixation System KF80 (Reichert-Jung, Vienna, Austria). Tissue blocks were moved by a precooled forceps. For freeze-substitution, tissue samples were immersed in a solution of 0.5% uranyl acetate in anhydrous methanol over night at −90 °C. The temperature was raised stepwise in 4 °C increments per hour from −90 to −45 °C, where it was kept for the subsequent steps. Tissue samples were washed several times with anhydrous methanol to remove residual water and uranyl acetate. The infiltration in Lowicryl HM20 went stepwise from Lowicryl/methanol 1:2, 1:1 and 2:1 (1 h each) to pure Lowicryl (overnight). For polymerization, the tissue was placed in a precooled embedding mall. The polymerization was catalyzed by ultraviolet light at a wavelength of 360 nm for two days at −45 °C followed by one day at room temperature (∼21 °C). Ultrathin sections (80 nm) were cut with a diamond knife on a Reichert-Jung ultramicrotome and mounted on nickel grids using an adhesive pen (EMS, Hatfield, PA, USA).

Grids with the ultrathin sections were processed at room temperature in solutions containing 50 mM Tris–HCl buffer pH 7.4, 50 mM NaCl, and 0.1% Triton X-100 (TBST). Sections were first etched in sodium ethanolate (1 g NaOH per 10 ml 100% ethanol, dipped 1 s, rinsed 30 s in water), submerged in TBST containing 0.1% sodium borohydride and 50 mM glycine for 10 min, and washed 10 min in TBST, followed by blocking in TBST containing 2% human serum albumin (HSA) for 10 min. They were then incubated overnight with primary antibodies diluted in TBST containing 2% HSA.

Antibodies against AMPA receptor subunit GluR1 (dilution: 1 μg/ml), GluR2/3 (dilution: 2 μg/ml) and NMDAR1 (dilution: 2 μg/ml) mixed together with NMDAR2A/B (dilution: 5 μg/ml) were used. Goat anti-rabbit immunoglobulins coupled to 10 nm gold particles (GAR10; British BioCell International, Cardiff, UK) were diluted 1:20 in TBST with 2% HSA and spun at 1000 r.p.m. All antibodies were the same as used for immunoblotting, where they showed single bands (Fig. 1). The antibodies provided by Wenthold have been extensively characterized before (Wenthold et al., 1992; Petralia et al., 1994a,b). Ultrathin sections were contrasted in uranyl acetate and lead citrate, before they were observed in a Philips CM100 electron microscope. Pictures were taken at a primary magnification of 43,000×.

Quantitative immunogold analysis

Electron micrographs with clearly visible synapses were taken randomly in the stratum radiatum of CA1 as well as in the hilus of fascia dentata and the stratum lucidum of the adjacent part of CA3. Mf synapses were identified as large presynaptic terminals with densely packed small clear spherical vesicles, a few dense core vesicles and by forming multiple asymmetric synaptic specializations on postsynaptic dendritic spines. The ssc synapses were identified as small terminals forming asymmetric synapses on spines in the stratum radiatum of CA1. For each antibody and for each of the five DKO and five WT mice 20 micrographs were recorded, yielding a total of 30–60 individual synaptic sites with clearly defined postsynaptic densities (PSDs). The lengths (nm) of these membrane profiles were recorded and the densities of gold particles calculated (Bergersen et al., 2005). Gold particles situated within ±30 nm (i.e. approximately the same distance as the lateral resolutions of the immunogold method (Chaudhry et al., 1995; Nagelhus et al., 1998) from the midline of the membranes were included in the quantifications. All synapses that met the criteria above were included regardless of whether they had gold particles or not. To minimize the influence of zero values on the means and S.E.M. in the individual animals, data for three syn-

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