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Munc13 genotype regulates secretory amyloid precursor protein processing via postsynaptic glutamate receptors

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ABSTRACT

The amyloid precursor protein (APP) can be proteolytically degraded via non-amyloidogenic α-secretase and amyloidogenic β-secretase pathways. Previously, we have identified the presynaptic protein Munc13-1 as a diacylglycerol/phorbolester (DAG/PE) receptor that contributes to secretory, non-amyloidogenic APP processing after PE stimulation. Here, we used organotypic brain slice cultures from wild-type mice and from Munc13-1 knock-out (KO), Munc13-2 KO and Munc13-1/2 double KO (DKO) mice for pharmacological stimulation experiments. First, we demonstrate that neuronal populations and synaptic components important for secretory APP processing develop normally in organotypic brain slice cultures of all genotypes analyzed. Blockade of voltage-gated Na^+ channels by tetrodotoxin reduced the PE-stimulated secretory APP processing, whereas depolarization by high extracellular K^+ concentration evoked APP secretion. Additionally, the PE-stimulated APP secretion from Munc13-1 KO brain slices was significantly lower than that from wild-type brain slices. This effect was not observed in brain slices from Munc13-2 KO mice, which is consistent with the lower abundance and subpopulation-specific distribution of Munc13-2 in presynaptic elements. In Munc13-1/2 DKO brain slices, the deficiency of Munc13-1 dominated the effect of APP processing. The Munc13-1 KO effect on APP processing could be rescued by the stimulation of postsynaptic glutamatergic receptors. This indicates that lack of postsynaptic glutamate receptor stimulation in Munc13-1 KO brain slice cultures but not presynaptic mechanisms account for compromised APP processing. We conclude that organotypic brain slices cultures are a valuable tool for studying APP processing pathways in intact neuronal circuits and that neuronal activity is important for maintenance of the non-amyloidogenic APP processing.

1. Introduction

Aberrant formation of β-amyloid peptides from transmembrane amyloid precursor protein (APP) is regarded as a key mechanism in the pathogenesis of Alzheimer's disease (Hardy and Selkoe, 2002; Querfurth and LaFerla, 2010). Normal physiological processing of APP is characterized by the balanced action of two competing secretory pathways, involving lumenal cleavage by α- and β-secretases, respectively (Hung et al., 1993; Skovronsky et al., 2000), followed by transmembraneous γ-secretase processing (Selkoe, 2001). Proteolytic cleavage within the β-amyloid sequence of APP by α-secretase precludes the generation of β-amyloid and leads to secretion of a protein with neuroprotective functions termed $APPs_\alpha$ (Sisodia, 1992; Thinakaran and Koo, 2008). This so-called ectodomain shedding is constitutively catalyzed by ADAM10 (Lammich et al., 1999; Kuhn et al., 2010), a member of the *a disintegrin and metalloprotease* family ADAM.

Disturbances of the equilibrium between the α- and β-secretory proteolytic pathways may lead to increased formation of β-amyloid, the main constituent of senile plaques and may thus become pathogenic. It is generally accepted that the non-amyloidogenic α-secretory pathway is partially regulated by members of the protein kinase C (PKC) family (Checler, 1995) via intracellular diacylglycerol (DAG) generation. Pharmacologically, increase in α-secretase processing of APP can be stimulated by phorbol esters (PE) which bind to and activate PKC resulting in decreased generation of β-amyloid (Buxbaum et al., 1990, 1993; Caporaso et al., 1992). Recently, we identified another DAG/PE receptor, Munc13-1 (Betz et al., 1998), as being an alternative presynaptic regulator of secretory APP processing in neurons and in mouse brain slices (Roßner et al., 2004). Munc13-1 is ubiquitously expressed in brain (Augustin et al., 1999a), and organotypic brain slices from Munc13-1 deletion mutant and dysfunctional Munc13-1 (H567K) knock-in mutant mice have been shown to release

Abbreviations: APP, amyloid precursor protein; DIV, days in vitro; DKO, double knock-out; KO, knock-out; TTX, tetrodotoxin.

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significantly less $APPs_{\alpha}$ in response to PE treatment compared to slices from wild-type mice (Roßner et al., 2004).

Proteins of the Munc13 family consist of two more isoforms, Munc13-2 and Munc13-3, which in mouse are restricted to neocortex/hippocampus and cerebellum, respectively (Augustin et al., 1999a). Munc13 proteins are localized to active zones of presynaptic elements and, functionally, play an essential role in vesicle maturation and enhance neurotransmitter release (Betz et al., 1998, Brose et al., 2000). Eliminated function of Munc13 and its homologues Unc13 (*C. elegans*) and Dunc13 (*D. melanogaster*), respectively, results in arrest of transmission in affected synapses (Aravamudan et al., 1999; Augustin et al., 1999b; Richmond et al., 1999). Additionally, a function of Munc13-1 in the induction of presynaptic LTP in hippocampal mossy fibers has recently been postulated (Yang and Calakos, 2011).

In glutamatergic mouse hippocampal neurons 90% of the synapses are solely Munc13-1-dependent while only 10% express the Munc13-2 isoform (Rosenmund et al., 2002). This results in a 90% reduction of glutamate release from Munc13-1 KO primary neurons (Augustin et al., 1999b). On the contrary, synapses of inhibitory GABAergic neurons were shown to redundantly contain both Munc13 isoforms (Varoqueaux et al., 2002). Thus, neuronal subpopulation- and synapse-specific distribution of Munc13 isoforms reflects differential control of neurotransmitter release (Rosenmund et al., 2002).

The impaired glutamate release can be attributed to the fact that vesicles in Munc13-1 deficient synapses are unable to gain fusion competence following docking to the presynaptic membrane (Augustin et al., 1999b). The remaining glutamate release reflects Munc13-2 function which is present in only a small fraction of cortical and hippocampal glutamatergic synapses (Varoqueaux et al., 2002; Rosenmund et al., 2002). Consequently, the release of glutamate is completely blocked in cerebral neurons of Munc13-1/2 DKO mice (Varoqueaux et al., 2002).

In this study, we sought to determine the relative contribution of synaptic activity on PE-stimulated APP processing and to investigate a possible involvement of neuronal activity in general and postsynaptic mechanisms in particular in the stimulated release of neuroprotective APP. Therefore, organotypic cultures from wild-type, Munc13-1 KO, Munc13-2 KO and Munc13-1/2 DKO mice were established in order to measure APP secretion following pharmacological manipulation of pre- and postsynaptic processes. We report a role of Munc13 proteins, of neuronal activity and of postsynaptic glutamate receptors in the PE-stimulated APP processing.

2. Experimental procedures

2.1. Mutant mice

The generation of mice deficient in Munc13-1, in Munc13-2 or both presynaptic proteins (Munc13-1/2 DKO) and genotyping protocols were published previously (Augustin et al., 1999a,b; Rhee et al., 2002). Heterozygous Munc13-1 KO and Munc13-1/2 DKO founder mice were generously provided by Nils Brose (Max Planck Institute for Experimental Medicine, Göttingen, Germany).

2.2. Organotypic brain slice cultures

This experimental study had institutional approval from the Landesdirektion Leipzig, license # T81/10 and was carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Organotypic brain slice cultures were established from wild-type C57Bl6 mice, from Munc13-1 KO and Munc13-2 KO mice and from Munc13-1/2 DKO mice as described recently (Roßner et al., 2004). Since Munc13-1 KO and Munc13-1/2 DKO mice have a lethal perinatal phenotype (Augustin et al., 1999b; Rhee et al., 2002) brains were prepared within 3 h after birth, mounted in 1.5% agarose in distilled water and 350 μm thick sections were cut in the coronal plane using a vibratome. Brain sections were collected and maintained in culture plate inserts (Millicell CM, 30 mm diameter; Millipore, Bedford, MA) in D-MEM/Ham's F12 medium containing 25% horse serum, 0.2% D-glucose, 2 mM L-glutamine and 0.3 mM HEPES. Slice cultures were allowed to develop for up to 21 days in vitro (DIV). At this culture age basic neuronal maturation such as development of perineuronal nets is accomplished in vitro (Gähwiler et al., 2001; Reimers et al., 2007). The medium was changed every other day and supplemented with inhibitors/blockers as indicated below. The conditioned medium was collected and stored in aliquots at −20 °C pending LDH activity assays.

2.3. LDH release assay

LDH released into the culture medium during the cultivation of untreated and treated wild-type and Munc13-1 KO, Munc13-2 KO and Munc13-1/2 DKO mouse brain slices was measured using the CytoTox non-radioactive cytotoxicity assay according to the manufacturer's protocol (Promega, Mannheim, Germany). To account for differences in the amount of cultured tissue per well, LDH was measured in aliquots of the conditioned medium during the whole cultivation period and in the brain slices at the end of the cultivation (day 15). The total amount of LDH released per well was added to the amount of LDH present in the brain slices of the same well and set to 100%. From this total amount of LDH present the relative amount of LDH sequentially released per 48 h of cultivation was calculated and given in percent.

2.4. Immunohistochemical characterization of organotypic brain slice cultures

At the end of the 15 days cultivation period brain slices were fixed with 4% paraformaldehyde in 10 mM phosphate buffer (pH 7.4) supplemented with 2% sucrose and stored in PBS at 4 °C. The following primary antibodies and lectins were used for immunohistochemical labelings: rabbit antibodies against the synaptic vesicle marker protein SV2 (Developmental Studies Hybridoma Bank; University of Iowa; 1:50), mouse antibodies against Munc13-1 (Synaptic Systems; Göttingen, Germany; 1:200), monoclonal mouse antibodies against the Ca^{2+}-binding protein parvalbumin (Swant, Bellinzona, Switzerland; 1:400), goat anti-choline acetyltransferase (ChAT; Millipore; Schwalbach, Germany; 1:400), rabbit anti-neuronal nitric oxide synthetase (nNOS; Transduction Laboratories; Heidelberg; Germany; 1:200) and a monoclonal antibody against intermediate neurofilaments (SMI311; Sternberg Monoclonals; Lutherville, MD; 1:500). Glycan components of perineuronal nets were visualized with biotinylated *Wisteria floribunda* agglutinin (bio-WFA, Sigma; 1:200) followed by its detection with Cy3-conjugated streptavidin (Sigma, Deisenhofen, Germany; 1:100). WFA lectin is known to detect N-acetylgalactosamine components and was recently shown that these detected components are highly dependent on the presence of aggrecan core protein, the main protein component of perineuronal nets (Giamanco et al., 2010). Alternatively, a rabbit anti-mouse aggrecan antibody (AB1031, Chemicon; 1:500) was used to detect the proteoglycan core protein component of perineuronal nets. For double labeling experiments cocktails of primary antibodies were incubated overnight in TBS containing 0.1% Triton X-100, 2% bovine serum albumine and 5% normal goat serum. After washing steps (3 times for 5 min in TBS) bound primary antibodies were visualized with Cy2- or Cy3-conjugated goat anti-rabbit or goat anti-mouse IgG (Dianova, Hamburg, Germany; 1:200) resulting in green or red fluorescent labeling, respectively. In control experiments, primary antibodies were omitted and sections were incubated with fluorochromated secondary antibodies.

2.5. Confocal laser scanning microscopy

Laser scanning microscopy (LSM 510, Zeiss, Oberkochen, Germany) was performed to reveal the presence and co-localization of neuronal structures. For the Cy2-labeled antigens (green fluorescence), an argon laser with 488 nm excitation and 510 nm emission was used applying a low-range band pass (505–530 nm). For Cy3-labeled antigens (red fluorescence), a helium–neon-laser with 543 nm excitation and 570 nm emission was used applying a high-range band pass (560–615 nm).

2.6. Pharmacological treatments of brain slice cultures

After 15 DIV, organotypic brain slice cultures of different Munc13 genotypes were stimulated with PE (Calbiochem, 100 nM) or vehicle (DMSO) for 6 h. In order to study the influence of neuronal activity on APP secretion, wild-type slice cultures were supplied with fresh medium containing 10 mM K^+ at 15 DIV. After 48 h the medium was removed and stored at −80 °C as an internal control of APP secretion in each culture well. Then, culture medium was replaced by medium containing decreased, $[K^+]_{3.6\,mM}$ ($n = 12$) or increased potassium concentrations, $[K^+]_{25\,mM}$ ($n = 10$) and $[K^+]_{50\,mM}$ ($n = 7$), respectively. Following additional 48 h the medium was again removed and stored. To block voltage-gated Na^+ channels during PE stimulation, 1 μM tetrodotoxin (TTX) was added in some experiments. Blockade of presynaptic glutamate release was achieved by blocking N- and P/Q-type Ca^{++} channels with (i) riluzole (100 μM) or (ii) with ω-conotoxin (1 μM) plus ω-agatoxin (500 nM) or (iii) with 10 μM conotoxin MVIIC. Glutamate receptors were blocked by treatment with a combination of MCPG (0.5 mM), a non-selective metabotropic glutamate receptor antagonist, and kynurenic acid (3.0 mM), a broad spectrum ionotropic glutamate receptor antagonist, 18 h prior to and during PE stimulation for 6 h. Alternatively, to activate postsynaptic glutamate receptors in Munc13-1 KO slice cultures during the PE stimulation glutamate (500 μM) was added to the cultivation medium. To block neurotransmitter release from inhibitory GABAergic synapses, tetanus toxin (20 nM) targeting the SNARE complex was applied during PE stimulation.