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Endogenous APP accumulates in synapses after BACE1 inhibition

Saket Milind Nigam[a], Shaohua Xu[a], Frauke Ackermann[a,1], Joshua A. Gregory[a,2], Johan Lundkvist[b], Urban Lendahl[c], Lennart Brodin[a,*]

[a] Department of Neuroscience, Karolinska Institutet, S-171 77 Stockholm, Sweden
[b] AlzeCure, Karolinska Institutet Science Park, S-141 57 Huddinge, Sweden
[c] Department of Cell and Molecular Biology, Karolinska Institutet, S-171 77 Stockholm, Sweden

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ABSTRACT

BACE1-mediated cleavage of APP is a pivotal step in the production of the Alzheimer related Aβ peptide and inhibitors of BACE1 are currently in clinical development for the treatment of Alzheimer disease (AD). While processing and trafficking of APP has been extensively studied in non-neuronal cells, the fate of APP at neuronal synapses and in response to reduced BACE1 activity has not been fully elucidated. Here we examined the consequence of reduced BACE1 activity on endogenous synaptic APP by monitoring N- and C-terminal APP epitopes by immunocytochemistry. In control rodent primary hippocampal neuron cultures, labeling with antibodies directed to N-terminal APP epitopes showed a significant overlap with synaptic vesicle markers (SV2 or synaptotagmin). In contrast, labeling with antibodies directed to C-terminal epitopes of APP showed only a limited overlap with these proteins. In neurons derived from BACE1-deficient mice, and in control neurons treated with a BACE1 inhibitor, both the N-terminal and the C-terminal APP labeling overlapped significantly with synaptic vesicle markers. Moreover, BACE1 inhibition increased the proximity between the APP C-terminus and SV2 as shown by a proximity ligation assay. These results, together with biochemical observations, indicate that BACE1 can regulate the levels of full-length APP at neuronal synapses.

1. Introduction

Synapses are sites of early pathological changes in Alzheimer disease (AD) and loss of synapses correlates strongly with cognitive deficits in AD (Serrano-Pozo et al., 2011). The exact cause of the synaptic impairment is unclear, but many lines of evidence indicate that amyloid β peptides (Aβ) derived from the proteolytic processing of the amyloid precursor protein (APP) plays a central role (Haass et al., 2012; Jonsson et al., 2012; Selkoe et al., 2012). APP is a transmembrane protein expressed at high levels in the brain and processed in a rapid and complex fashion. In the amyloidogenic pathway sequential cleavages by β- and γ-secretases generate amyloid β peptide (Aβ), which has been linked with pre- and postsynaptic derangements. While inhibitors of γ-secretase aimed for AD have precluded further development, several β-secretase inhibitors have reached late stage clinical trials. Interestingly, β-secretase cleavage of APP, unrelated to Aβ formation, has also been implicated in synaptic impairment (Gouras et al., 2010; Mucke and Selkoe, 2012; Tamayev et al., 2012). It is thus of considerable interest to elucidate APP biology at the level of the synapse and how it might be affected by altered BACE1 activity.

While the trafficking and processing of APP has been explored in great detail in non-neuronal cells, knowledge about the fate of APP in neurons and neuronal synapses is less complete (Haass et al., 2012). Moreover, it has proven difficult to determine the correlation between the behavior of expressed tagged APP constructs and that of endogenous APP in neurons (Groemer et al., 2011; Kohli et al., 2012; Rodrigues et al., 2012; Sannerud et al., 2011; Villegas et al., 2013). The present study focused on examining endogenous APP at hippocampal synapses. We used immunocytochemistry and proximity ligation assay (PLA) to examine whether deficiency or inhibition of the main β-secretase BACE1 would influence the pattern of N- and C-terminal APP epitopes at synapses.

* Corresponding author. Tel.: +46 8 524 869 02; fax: +46 8 32 58 61.
E-mail address: lennart.brodin@ki.se (L. Brodin).
[1] Present address: Deutsches Zentrum fur Neurodegenerative Erkrankungen (DZNE), Charitéplatz 1, D-10117 Berlin, Germany.
[2] Present address: Institute of Environmental Medicine, Karolinska Institutet, S-171 77 Stockholm, Sweden.

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2. Materials and methods

2.1. Neuron culture

All animal experiments were approved by the North Stockholm Ethics Committee. Sprague Dawley rats, C57/BL6J mice, and BACE1 knockout mice (Roberds et al., 2001) of either sex were used. Offspring were sacrificed at P0 or P1. Hippocampi were treated with trypsin (0.25%, Sigma) for 7 min at 37 °C and re-suspended in complete Neurobasal medium supplemented with 2% B27, 1% L-glutamine and 1% penicillin/streptomycin (Gibco). Cells were passed through a 70 μm filter (BD Biosciences) and plated at a density of approximately 25,000 cells/well on poly-L-coated cover slips. Five μM cytosine β-D-arabinofuranoside was added on 2 DIV and cells were grown for 14 days changing half of the medium every 2 days. The BACE1 inhibitors AZ20 (AstraZeneca AB) (Mattsson et al., 2012) and C3 (Calbiochem Millipore) (Stachel et al., 2004) were used at concentrations of 0.5 μM and 2 μM, respectively.

2.2. Antibodies

Multiple antibodies recognizing different epitopes of APP were used: anti-APP 22C11 (here termed "N-t1", mouse monoclonal, Millipore) recognizing aa 66–81 of APP; anti-APP A8967 ("N-t2", rabbit polyclonal, Sigma) recognizing aa 46–60 of APP; anti-APP CT695 ("C-t1", rabbit polyclonal, Invitrogen) raised against a 22 amino acid peptide from the C-terminus of APP; anti-APP Y188 ("C-t2", rabbit monoclonal, Epitomics) recognizing the YENPTY motif in the C-terminus of APP. A study of antibody specificity found that the C-t2 antibody is highly specific for APP, whereas some non-specific labeling was present for other APP antibodies (Guo et al., 2012). At present we have been unable to find a highly specific antibody recognizing the N-terminal of APP and thus some degree of non-specific labeling could not be avoided. Pan-anti-SV2 (mouse monoclonal) (Buckley and Kelly, 1985), anti-synaptotagmin 1 (rabbit polyclonal) (Jakobsson et al., 2011), anti-VAMP2 (Jakobsson et al., 2008), and anti-PSD95 (mouse monoclonal, Synaptic Systems) antibodies were used to label synapses.

2.3. Immunocytochemistry

Neuron cultures with a sparse neurite network were fixed for 20 min in 4% paraformaldehyde, 0.1 M phosphate buffer pH 7.4 with 4% sucrose. After washing, cells were incubated for 20 min in PBS containing 0.3% Triton X-100, followed by 30–60 min blocking in 5% normal goat serum (NGS) and 0.1% Triton X-100 in PBS. Cover slips were incubated with primary antibodies (diluted in 3% NGS and 0.1% Triton X-100 in PBS) over night at 4 °C, followed by 1.5 h incubation at RT with Alexa-conjugated secondary antibodies (anti-rabbit Alexa488/555; anti-mouse Alexa488/555, Invitrogen), and mounted in VectaShield mounting medium (Invitrogen). Images were acquired using a Zeiss 510 laser scanning microscope and a 63×, 1.4 N.A. objective (Zeiss). Argon and HeNe1 lasers were used to excite fluorophores at 488 nm and 555 nm wavelengths, respectively. Laser power and pinhole were adjusted for each channel separately before each microscope session. The fluorescence detection was then adjusted by changing only the detector gain and amplifier offset. Regions containing axonal profiles (as judged by the SV2/synaptotagmin staining pattern) were imaged at 2× and 4× digital zoom. Images were exported using Zeiss LSM Image Browser software.

2.4. Image analysis

(1) In the "*threshold test*" the degree of colocalization between SV2/synaptotagmin and APP labeling was analyzed at 2× zoom using the ImageJ (NIH, USA) plugin Colocalization highlighter. A threshold was set at a given intensity value and kept constant throughout the analysis. For pixel intensity values above the threshold, colocalized points from both channels (red-SV2/synaptotagmin, green-APP) were shown in white as a separate image. Synaptic terminals shown in white were counted along with terminals positive for SV2/synaptotagmin alone. For each experimental condition 300–500 nerve terminals were collected. (2) In the "*ratio test*", the ratio between the APP epitope fluorescence within a synapse ("ROI1", defined by the shape of the SV2/synaptotagmin labeling and marked using the ImageJ oval selection tool) and the fluorescence in an adjacent axonal region ("ROI2") was calculated. To define ROI2, one peri-synaptic ROI (identical in shape to ROI1) was placed on each side of ROI1 along the length of the axon. The mean value of the fluorescence intensities in the two peri-synaptic ROIs was defined as the intensity in ROI2. Only synapses distinctly separated from adjacent synaptic regions were included (>30 synapses for each condition) and analyzed at 4× zoom. Each experimental condition was tested in a minimum of 3 separate cultures prepared on different days.

2.5. Proximity ligation assay

In situ proximity ligation assay (PLA) (Soderberg et al., 2008) was performed with the Duolink system using Detection Reagents Orange (Olink Bioscience). Cells were fixed, permeabilized and blocked as described above. Following overnight incubation with primary antibodies (C-t2 and SV2; positive control VAMP2 and SV2; negative control SV2 alone) at 4 °C, coverslips were incubated with oligonucleotide-conjugated antibodies: PLUS-anti-rabbit PLA probe and MINUS-anti-mouse PLA probe. After ligation, the oligonucleotide strands were amplified with polymerase, followed by hybridization with fluorescent oligonucleotides. All incubations were done at 37 °C using the incubation times and buffers specified by the manufacturer (Olink Bioscience) with three exceptions: the probe incubation time, the ligation time, and the amplification time were increased by 30 min. In addition, following the PLA reaction, an Alexa488-conjugated synaptotagmin antibody was applied at room temperature for 90 min to label synapses. BACE1 inhibitor data was normalized to control data for each experiment. No PLA signals were detected in the negative controls. For quantification, 4× zoom images were loaded onto a CellProfiler pipeline for automated analysis. Synapses were identified based on a global thresholding of SV2 labeling using the Otsu method as well as size parameters. The size of these synapses was adjusted in accordance with positive control for the PLA reaction. Thereafter, PLA signals were identified using the same thresholding strategy and size parameters. The two channels were related and the number of synapses positive for PLA signals was determined. For each condition 150–300 synapses were quantified.

2.6. Western blot analysis

For analysis of full-length APP, brains of 3 adult mice of each genotype were homogenized in buffer (4 mM HEPES, pH 7.4; 0.32 M sucrose; 1% SDS; 1 mM PMSF and protease inhibitor cocktail, Sigma). Protein concentrations were determined by NanoDrop 2000 (Thermo). Equal amounts of protein from each homogenate were resolved by SDS-PAGE using 10% NuPAGE Bis-Tris Gel (Invitrogen) and transferred onto PVDF membranes (Millipore). To better resolve C-terminal fragments (CTFs) a 16% Tricine Gel (Novex) was used; two adjacent bands of approximately 10–12 kD were identified, with the upper band presumably corresponding to β-CTF and the lower band corresponding to α-CTF. After blocking in 5% non-fat milk in TBS containing 0.1% Tween 20, the membranes were incubated with primary antibodies at 4 °C overnight. Membranes