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Major amyloid-β–degrading enzymes, endothelin-converting enzyme-2 and neprilysin, are expressed by distinct populations of GABAergic interneurons in hippocampus and neocortex

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ABSTRACT

Impaired clearance of amyloid-β peptide (Aβ) has been postulated to significantly contribute to the amyloid accumulation typical of Alzheimer's disease. Among the enzymes known to degrade Aβ in vivo are endothelin-converting enzyme (ECE)-1, ECE-2, and neprilysin (NEP), and evidence suggests that they regulate independent pools of Aβ that may be functionally significant. To better understand the differential regulation of Aβ concentration by its physiological degrading enzymes, we characterized the cell and region-specific expression pattern of ECE-1, ECE-2, and NEP by in situ hybridization and immunohistochemistry in brain areas relevant to Alzheimer's disease. In contrast to the broader distribution of ECE-1, ECE-2 and NEP were found enriched in GABAergic neurons. ECE-2 was majorly expressed by somatostatin-expressing interneurons and was active in isolated synaptosomes. NEP messenger RNA was found mainly in parvalbumin-expressing interneurons, with NEP protein localized to perisomatic parvalbuminergic synapses. The identification of somatostatinergic and parvalbuminergic synapses as hubs for Aβ degradation is consistent with the possibility that Aβ may have a physiological function related to the regulation of inhibitory signaling.

1. Introduction

It is widely believed that impaired clearance of amyloid-β peptide (Aβ) contributes significantly to the abnormal accumulation and aggregation of Aβ characteristic of Alzheimer's disease (AD) (Selkoe and Hardy, 2016). As a product of the physiological processing of amyloid-precursor protein (APP) by β- and γ-secretases, Aβ is produced continually throughout the lifespan, and its concentration is tightly controlled by the activities of several proteases (Baranello et al., 2015). The half-life of the peptide in brain is between 0.5 and 3 hours (Basak et al., 2012), and disruption of the activities of Aβ degrading proteases through pharmacological inhibition or genetic inactivation results in increased steady-state levels of endogenous Aβ in the brains of mice (Pacheco-Quinto et al., 2013). This complex regulation of both Aβ production and degradation strongly supports a key physiological function for the peptide, and mounting evidence indicates that Aβ is important for synaptic plasticity and cognition (reviewed in Puzzo et al., 2015). The enzymes responsible for degrading Aβ could therefore be viewed as regulators of normal Aβ function and would be expected to concentrate in the microenvironments where Aβ fulfills its function. Therefore, establishing the specific cell-type distribution of these enzymes in the brain may yield insights on Aβ's physiological role.

Among the enzymes known to degrade Aβ are several members of the M13 family of type II integral membrane zinc metalloproteases, including endothelin-converting enzyme (ECE)-1, ECE-2, neprilysin (NEP) and NEP-2 (reviewed in Saido and Leissring, 2012). Mice deficient in each of these enzymes have elevated Aβ levels, and although they are highly related, the activities of the ECEs and NEP are unable to compensate for one another's loss of function, suggesting that they regulate independent pools of Aβ (Eckman et al., 2006). The apparent compartmentalization of Aβ degradation could be a reflection of the different subcellular localization of Aβ-degrading enzymes. For instance, NEP localizes mainly to the plasma membrane, has a neutral pH optimum, and degrades Aβ in the extracellular space (Iwata et al., 2000; Shirotani et al., 2001), whereas ECEs are active in acidic Aβ-producing intracellular compartments, either in early endosomes before

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secretion or in the endosomal/lysosomal pathway (Eckman et al., 2001; Pacheco-Quinto and Eckman, 2013). This compartmentalization of Aβ catabolism may also result from differences in the expression pattern of the enzymes. Therefore, to better understand the nonoverlapping roles of the ECEs and NEP in regulating physiological Aβ concentration and preventing its aggregation in vivo, we characterized the cell and region-specific expression pattern of *Ece1*, *Ece2*, and *Mme* (neprilysin, also known as membrane metalloendopeptidase) in brain areas relevant to AD.

2. Materials and methods

2.1. Mice

Mice were housed in ventilated microisolator cages with free access to food and water and were maintained at 25 °C with a 12/12 hours light/dark cycle. Three-month-old B6C3F1 mice (strain code 031, Charles River Laboratories) and TgCRND8 mice (kindly gifted by Dr. Paul Fraser, University of Toronto) were euthanized by CO_2 asphyxiation as approved by the Institutional Animal Care and Use Committee at Rutgers University and consistent with American Veterinary Medical Association guidelines.

2.2. Tissue processing

Immediately after euthanasia, brains were quickly dissected and frozen on dry ice or immersed in 10% neutral buffered formalin for 24 hours and then dehydrated and embedded in paraffin blocks. Coronal sections, 5-μm thick, were air dried overnight, baked for 60 minutes at 60 °C, and stored at −20 °C until use.

2.3. Fluorescence in situ hybridization

ViewRNA dual in situ hybridization (ISH) from Affymetrix (Santa Clara, CA, USA) was performed according to the manufacturer's instructions with a 10-minute boil at 95 °C in pretreatment solution and 20-minute incubation with protease solution. The probe sets used for the study were *Sst* (NM_009215, region covered 20-549), *Mme* (NM_008604; region covered 114-1082), *Ece-1* (NM_199307, region covered 1200-2161), *Ece-2* (NM_177941, region covered 1501-2413), *Gad1* (NM_008077, region covered 154-2441), *Gfap* (NM_010277, region covered 23-1144), and *Gapd* (NM_008084, region covered 109-1028). The ISH probe sets used to detect *Mme*, *Ece1*, and *Ece2* are expected to bind to all known transcript variants, with the exception of *Ece2* variant 3 (NM_025462.2), which lacks a protease domain and was deliberately excluded from the study. Nuclei were visualized with the fluorescent dye Hoechst 33258 (AnaSpec, Fremont, CA, USA). Specificity of *Ece2* and *Mme* probe sets was assessed using archived tissue from ECE-2 knockout mice (Yanagisawa et al., 2000) and NEP knockout mice (Lu et al., 1995), and their wild-type littermates (Supplementary Fig. 1).

2.4. Immunohistochemistry

Tissue sections were blocked with 5% BSA containing 0.1% triton X-100 and then incubated sequentially with primary antibodies and AlexaFluor conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). Primary antibodies used included rat monoclonal anti-somatostatin (1:100, Millipore MAB354), rabbit polyclonal anti-parvalbumin (1:1000, Abcam ab11427), rat monoclonal anti-neprilysin (1:100, R&D Systems mAB1126), mouse monoclonal anti-CD31/PECAM1 (Novus Biologicals NB100-64796), and mouse monoclonal anti-GFAP (1:100, Novus Biologicals NBP1-05197). Immunofluorescence was visualized with a Zeiss Axio Imager Z1 fluorescent microscope. For combined in situ hybridization and immunohistochemistry, immunodetection was conducted after in situ hybridization was completed.

2.5. Synaptosome preparation

Following a protocol developed by Dunkley et al. (Dunkley et al., 2008), mouse brains were homogenized with 10 strokes of a Dounce homogenizer in 0.32-mM sucrose, 5-mM Tris pH 7.4, 25-μM dithiothreitol containing 1X protease inhibitor cocktail without EDTA (Thermo Scientific), at a concentration of 20 mg/mL (w/v). Samples were centrifuged for 10 minutes at 2000 rpm in a Beckman bench top centrifuge, and 2.5 mL of the supernatant was layered on top of a discontinuous 0-3-10-15-23% Percoll gradient and centrifuged for 5 minutes at 20,000 rpm using a Sorvall SS-34 rotor. Synaptosomes were collected from the interface between the 15% and 23% layers and pelleted by centrifugation at 13,000 rpm. Protein concentration was determined by BCA assay (Thermo Scientific Pierce).

2.6. Big endothelin-1 conversion assay

Twenty-five micrograms of protein from synaptosomal preparations were incubated with 100-nM recombinant human big endothelin (ET)-1 (American Peptide Company) in 0.1-M sodium citrate buffer (pH 5.5) containing 0.05% Triton X-114. Protease inhibitor cocktail without EDTA (Thermo Scientific) and 100-nM thiorphan were added to inhibit the degradation of ET-1 by NEP and other peptidases. After 1 hour incubation at 37 °C, reactions were stopped by adding EDTA (5-mM final concentration), and levels of ET-1 were measured by sandwich ELISA (R&D systems, Minneapolis, MN, USA).

3. Results

3.1. Ece2 *and* Mme*, but not* Ece1*, are selectively expressed by GABAergic neurons*

Single and multiplex in situ hybridization (ISH), employing branched DNA signal amplification for single-molecule sensitivity, were used to overcome several of the challenges associated with analysis of genes expressed at low levels in the brain. The initial ISH results for the ECEs and NEP (*Mme*) were quite striking and unexpected; despite the substantial contribution of ECE-2 and NEP to overall brain Aβ levels (Eckman et al., 2006, 2003; Iwata et al., 2001), the messenger RNA (mRNA) for both enzymes was found enriched in a sparse number of cells throughout the cortex as well as organized through the parenchyma in a pattern resembling neural processes (Fig. 1). For *Ece2*, this nonsomatic fiber-like pattern extended to layer I, where no *Ece2*+ cells were found, possibly indicating that *Ece2* mRNA travels through processes from deeper cortical layers. Based on the distribution of *Ece2* and *Mme* positive cells, reminiscent of GABAergic interneurons, we performed dual ISH with the GABA-synthetic enzyme, glutamic acid decarboxylase-67 (*Gad1*). Nearly all *Ece2*+ and *Mme*+ cells coexpressed *Gad1*, indicating that subpopulations of GABAergic interneurons are highly enriched in the expression of these Aβ-degrading enzymes.

In contrast to *Ece2* and *Mme*, *Ece1* mRNA was not particularly enriched in the soma of neurons and colocalized with *Gad1* only occasionally (Fig. 2A). Consistent with ECE-1's well-established function of generating the vasoactive peptide endothelin-1 (Xu et al., 1994), a portion of *Ece1* mRNA colocalized with cells immunopositive for platelet endothelial cell adhesion molecule 1 (PECAM-1, also known as CD31), an endothelial cell marker (Fig. 2B). In addition to the vasculature, *Ece1* mRNA was also found in a neuronal process-like pattern. Because *Ece1* did not appear to