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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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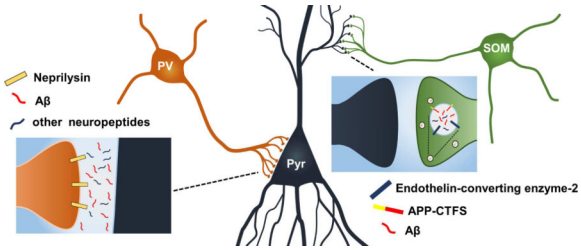
Abstract

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, 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 neprilysin 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 neprilysin were found enriched in GABAergic neurons. ECE-2 was majorly expressed by somatostatin-expressing interneurons and was active in isolated synaptosomes. Neprilysin mRNA was found mainly in parvalbumin-expressing interneurons, with neprilysin 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.

Graphical abstract



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Author contributions

J.P.Q and E.A.E designed the study, J.P.Q performed the experiments, and J.P.Q, C.B.E, and E.A.E interpreted the findings and wrote the paper.

Keywords

Alzheimer's disease; Aβ degradation; endothelin-converting enzyme; GABA; interneuron; neprilysin; somatostatin; parvalbumin

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 though 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) while ECEs are active in acidic Aβ-producing intracellular compartments, either in early endosomes before 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 non-overlapping 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 metallo-endopeptidase) in brain areas relevant to AD.

2. Materials and methods

2.1 Mice

Mice were housed in ventilated micro-isolator cages with free access to food and water and were maintained at 25 °C with a 12/12 hour 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 AVMA 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 h and then dehydrated and embedded in paraffin blocks. Coronal sections, 5 μm thick, were air dried overnight, baked for 60 min at 60° C and stored at −20 °C until use.

2.3 Fluorescence in situ hybridization

ViewRNA™ dual 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 probesets 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* probesets 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 Figure 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). 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 ten strokes of a Dounce homogenizer in 0.32 mM sucrose, 5 mM Tris pH 7.4, 25 μM DTT containing 1× protease inhibitor cocktail without EDTA (Thermo Scientific), at a concentration of 20 mg/ml (w/v). Samples were centrifuged for 10 minutes at 2,000 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

25 μg 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 1h incubation at 37°C, reactions were stopped by adding EDTA (5mM final concentration) and levels of ET-1 were measured by sandwich ELISA (R&D systems, Minneapolis, MN).

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,Eckman, et al., 2003,Iwata, et al., 2001), the 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 (Figure 1). For *Ece2*, this non-somatic fiber-like pattern extended to layer I, where no *Ece2*+ cells were found, possibly indicating that ECE-2 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 (GAD67, *Gad1*). Nearly all *Ece2*+ and *Mme*+ cells co-expressed *Gad1*, indicating that subpopulations of GABAergic interneurons are highly enriched in expression of these Aβ degrading enzymes.

In contrast to *Ece2* and *Mme, Ece1* mRNA was not particularly enriched in the soma of neurons and co-localized with *Gad1* only occasionally (Figure 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 co-localized with cells immunopositive for platelet endothelial cell adhesion molecule 1 (PECAM-1, also known as CD31), an endothelial cell marker (Figure 2B). In addition to the vasculature, ECE-1 mRNA was also found in a

neuronal process-like pattern. Since *Ece1* did not appear to be expressed selectively by a certain cell type, subsequent experiments were focused on identifying the GABAergic neuron subtypes expressing *Ece2* and *Mme*.

3.2 Ece2 is selectively expressed by somatostatin positive interneurons

In rodents, GABAergic interneurons represent ~10–20% of all neurons in neocortex, with 40% expressing parvalbumin (PV, *Pvalb*), 30% expressing somatostatin (SOM, *Sst*), and most of the remaining cells expressing the ionotropic serotonin receptor 5HT3aR, with little overlap among the three categories (Rudy, et al., 2011). Using dual ISH, we found that the majority of *Ece2* mRNA in the cortex concentrated in the soma of cells positive for somatostatin (Figure 3), and an estimated 92% of *Sst*+ cells (from a total count of 738 cells) co-expressed *Ece2*. Remarkably, although *Sst* was expressed at substantially higher levels than *Ece2, Sst* mRNA was nearly all concentrated in the soma versus the wider distribution of *Ece2*. In light of the selective expression of *Ece2* by *Sst*+ neurons, and in agreement with the connectivity in layer I of ascending SOM+ axons with dendrites of pyramidal neurons (Kawaguchi and Kubota, 1996), the presence of *Ece2* mRNA in layer I (Figure 1C) suggests that *Ece2* transcripts are transported to the end terminals of SOM interneurons.

In hippocampus, different subpopulations of *Sst*+ cells exist, mainly residing in the *stratum oriens* of CA1–3 and the *hilus* of the dentate gyrus. Virtually all *Sst*+ cells in *stratum oriens* were positive for *Ece2* (Figure 3). A particular subtype of SOM interneurons in this region, known as oriens-lacunosum-moleculare (O-LM) cells, have horizontal dendrites in *stratum oriens* and perpendicular axons that project to the *stratum lacunosum-moleculare* (Somogyi and Klausberger, 2005). Combining ISH for *Ece2* and immunohistochemistry (IHC) for SOM showed fibers containing *Ece2* mRNA extending to the *stratum lacunosum-moleculare*, where both *Ece2* mRNA and SOM-immunopositive fibers were enriched (Figure 4). Of note, at the border of *stratum radiatum* and *stratum lacunosum-moleculare* we detected a few *Ece2*+ cells that did not co-express somatostatin. Interneurons in this region have not been thoroughly characterized neurochemically (Somogyi and Klausberger, 2005).

In *hilus*, as in *stratum oriens, Ece2* was enriched in cells that were *Sst*+ (Figure 3) as well as immunopositive for SOM (Figure 4). *Hilar* SOM+ interneurons contact dendrites of pyramidal cells in the outer molecular layer, where *Ece2* mRNA was also present. Interestingly, though the granule layer of the dentate gyrus was the area of hippocampus with the most *Ece2* signal (Figure 4), the transcripts did not appear to surround the nuclei of granule cells and instead followed a neuronal process-like pattern indistinguishable from that observed for *Gad1* (Supplementary Figure 2). This result suggests that *Ece2* might not be expressed by granule cells *per se*, but rather by GABAergic neurons projecting to granule cells or through the granule layer towards the molecular layer.

3.3 ECE-2 is active in synaptosomes

The apparent enrichment of *Ece2* mRNA in somatostatinergic fibers, evidenced by the detection of abundant *Ece2* mRNA in layer I and *stratum lacunosum-moleculare*, raises the possibility that *Ece2* transcripts may be transported to synapses for local translation. To determine whether ECE-2 activity is present in synapses, we measured the conversion of big

endothelin (ET)-1 to ET-1 by isolated brain synaptosomes at pH 5.5 (Emoto and Yanagisawa, 1995). The results of this selective assay for ECE-2 activity confirmed that the enzyme is present in synaptosomes (Figure 5).

3.4 Neprilysin expression is enriched in parvalbumin positive interneurons

Early immunohistological studies revealed that NEP is expressed in brain, particularly in striatum, consistent with the enzyme's role in neuropeptide metabolism (Matsas, et al., 1986,Wilcox, et al., 1989). Subsequent reports confirmed that NEP is present in multiple brain regions and is primarily neuronal (Facchinetti, et al., 2003,Fukami, et al., 2002,Gaudoux, et al., 1993,Li, et al., 1995). Though the specific neuronal subtypes expressing the enzyme were not determined, NEP immunoreactivity was found associated with both GABAergic and glutamatergic fibers (Fukami, et al., 2002). More recently, a study of metalloprotease expression in perineuronal net-enwrapped PV+ interneurons suggested the intriguing possibility that NEP may be enriched in cortical PV+ neurons (Rossier, et al., 2015). Guided by this, we combined ISH for *Mme* (neprilysin) with IHC for PV, and found that the cortical GABAergic cells expressing *Mme* were parvalbuminergic (Figure 6). Remarkably, we observed that in somatosensory cortex, NEP immunoreactivity was not prominently detected in PV+ neuronal cell bodies, but rather surrounding the soma of pyramidal cells, mainly in layer 5. PV+ neurons are the main type of perisomatic inhibitory interneurons (Freund and Katona, 2007), and as shown in Figure 7, PV+ fibers surrounding the soma of pyramidal cells were found associated with NEP immunoreactivity. Thus, based on the known presynaptic localization of NEP (Fukami, et al., 2002), restricted expression of *Mme* mRNA by PV+ neurons, and the enrichment of NEP protein around pyramidal cells with strong PV+ perisomatic innervation, it is likely that NEP is selectively enriched in presynaptic endings of PV axons. The fact that little-to-no NEP protein was detected in the soma of cortical PV+ interneurons indicates that the protein is specifically transported to the synaptic region (Iwata, et al., 2004) and/or that *Mme* transcripts are translated there.

In hippocampus, we detected a population of PV-immunopositive *Mme*-expressing cells along the *stratum pyramidale*, following the expected distribution for PV+ interneurons (Figure 8). In addition to the soma of PV+ cells, *Mme* transcripts were also detected in a fiber-like pattern concentrated especially in areas with non-somatic PV immunolabeling. Using double immunofluorescence, we found an enrichment of NEP protein immunoreactivity within the *stratum pyramidale*, an area with strong PV innervation, as well as the *stratum lacunosum moleculare* (Figure 9), confirming earlier results from Fukami et al. (Fukami, et al., 2002). While the *stratum lacunosum moleculare* is innervated by certain weakly PV-immunopositive O-LM neurons (Klausberger, et al., 2003), NEP positive fibers in this region may also belong to other undetermined neuronal populations.

3.5 Ece2 and Mme are not upregulated by reactive glia

Reactive astrocytes and microglia are a common finding surrounding amyloid plaques and are considered contributors to Aβ removal. Despite the specific expression of *Ece2* and *Mme* by interneurons, we questioned whether the ECEs and NEP may also be upregulated by activated glia. Thus, we evaluated expression of the enzymes in areas with active gliosis in brains from TgCRND8 APP transgenic mice with significant amyloid deposition. As

expected for a marker of reactive astrocytes, increased *Gfap* expression was found surrounding amyloid plaques and coincided with increased immunoreactivity for GFAP (Figure 10). However, there was no indication of increased expression of *Ece1, Ece2* or *Mme* in peri-plaque areas with high GFAP immunoreactivity.

4. Discussion

Our histological study of hippocampus and neocortex reveals that two of the major Aβ degrading enzymes, ECE-2 and NEP, are expressed almost exclusively by two non-overlapping populations of GABAergic interneurons. Aside from being the likely explanation for our previous observation that these enzymes cannot compensate for one another (Eckman, et al., 2006), our results underscore the role that somatostatinergic and parvalbuminergic neurons may have in normal Aβ physiology, as well as in the pathophysiology of AD.

SOM+ interneurons compose ~5–8% of the total neuronal population and although they vary greatly in electrophysiological properties, morphology and connectivity (Ma, et al., 2006), their inhibitory activity is fundamental for cognitive function. Demonstrated impairments in learning and memory in ECE-2 knockout mice (Rodriguiz, et al., 2008) suggest that ECE-2 activity may be critical for SOM+ interneuron function, though further studies are required to determine whether disturbances in the homeostasis of Aβ, and/or other potential ECE-2 substrates, can directly alter somatostatinergic neurotransmission.

In AD, there is a clear loss of SOM innervation (reviewed in (Martel, et al., 2012) and several studies with APP transgenic mice support that SOM+ cells may be highly vulnerable to pathological Aβ accumulation (Albuquerque, et al., 2015,Ma and McLaurin, 2014,Perez-Cruz, et al., 2011,Ramos, et al., 2006). The sensitivity of these specific interneurons to AD pathology may relate to their putatively high capacity for intracellular Aβ metabolism via expression of ECE-2. Given that ECE-2 degrades only intracellular Aβ (Pacheco-Quinto and Eckman, 2013), the ~30% increase in endogenous Aβ measured in whole brain homogenates from ECE-2 knockout mice (Eckman, et al., 2003) may be derived from locally high accumulation within SOM+ cells. Any impairment in ECE-2 activity, or imbalance between intracellular production and degradation, could conceivably result over time in the formation of intraneuronal Aβ aggregates, considered a first step in the amyloid cascade (Wirths, et al., 2004).

One interesting subgroup of SOM+ cells to consider, with respect to intracellular Aβ accumulation and the potential to propagate amyloid pathology, is long-range-projecting interneurons. Whereas interneurons, by definition, mainly modulate the function of local principal cells, a specialized group of GABAergic inhibitory neurons project to distant areas of the brain helping to coordinate activity across regions. More than 90% of hippocampal long-range interneurons projecting to the basal forebrain are SOM+ positive cells preferentially distributed along *stratum oriens* and *hilus* of dentate gyrus (Jinno and Kosaka, 2002). Due to their long projections, this type of interneuron must rely on efficient retrograde transport to ensure the optimal traffic of synaptic cargo to the soma. Since ECE-2 resides mainly in endosomal/lysosomal vesicles of the retrograde transport pathway,

disturbances in ECE-2 expression, or impairments in vesicular trafficking that prevent proper localization of ECE-2 activity, could lead to the formation and interregional dissemination of Aβ aggregates.

There is currently no consensus as to whether ECE-2 expression or activity are disrupted in AD. In a microarray study of gene expression in inferior parietal lobe, *ECE2* was found to be the single most down-regulated gene, with average decreases of ~75% in AD compared to non-demented controls (Weeraratna, et al., 2007). A subsequent study of ECE2 expression in temporal lobe found increased expression in AD (Palmer, et al., 2009). The contrasting results of these studies could reflect regional differences in SOM+ interneuron pathology in AD (Chan-Palay, 1987,Kowall and Beal, 1988). They also highlight the difficulties in interpreting the pathophysiological significance of markers detected in end-stage diseased tissue.

Clear evidence for impaired NEP expression or activity in AD is similarly lacking, with studies reporting conflicting results (Carpentier, et al., 2002,Gahete, et al., 2010,Miners, et al., 2009,Wang, et al., 2005). However, as for SOM+ interneurons, mounting evidence supports the link between PV+ interneuron dysfunction and AD. PV+ cells are the largest group of GABAergic interneurons and work as a network generating high-frequency oscillatory currents, known as gamma oscillations, which give temporal reference to pyramidal firing, thus synchronizing the activity of large neuronal groups (Cardin, et al., 2009). Disturbances in gamma oscillations are the suspected culprits of the spontaneous neuronal discharges, cognitive impairment and premature death typical of APP transgenic mice (Minkeviciene, et al., 2009,Verret, et al., 2012), and may be associated with the excitation-inhibition imbalance and susceptibility to seizures in AD (Goutagny and Krantic, 2013).

Studies of physiological Aβ regulation have shown that synaptic activity-mediated increases in Aβ may serve as a negative feedback mechanism to reduce neuronal firing (Cirrito, et al., 2005,Kamenetz, et al., 2003). Given the 2-fold increase in endogenous Aβ in NEP knockout mice (Iwata, et al., 2001), the large pool of Aβ degraded at PV+ synapses may represent an important component of this inhibitory feedback. Interestingly, a pool of endogenous Aβ regulated by NEP was recently shown to positively modulate synaptic vesicle release probability in the hippocampus (Abramov, et al., 2009). Considering that this specific Aβ pool may act locally, and that Aβ can influence gamma oscillatory power (Nerelius, et al., 2009), it is conceivable that Aβ's physiological function at PV+ synapses might be the modulation of PV+ synaptic transmission. As a consequence, dysfunction in NEP activity, or other disturbances that abnormally elevate Aβ in PV+ synapses, could disrupt gamma rhythms.

The concentration of NEP activity at PV+ synapses may be functionally important for cognition beyond regulation of Aβ. As indicated by one of its original names (enkephalinase), NEP participates in the termination of enkephalin signaling (Matsas, et al., 1984) as well as in the hydrolysis of other neuropeptides relevant for interneuron function including neuropeptide Y (Rose, et al., 2009), somatostatin (Barnes, et al., 1995,Sakurada, et al., 1990), cholecystokinin (Matsas, et al., 1984), and vasoactive intestinal peptide (Duggan,

et al., 1994). Among known NEP substrates, the metabolism of enkephalins in brain is particularly interesting in light of our results. Enkephalins are important facilitators of long term potentiation by lowering GABA release from PV+ neurons, via activation of μ-opioid receptors, which are enriched in hippocampal PV+ synapses (Drake and Milner, 2006).

The finding that NEP is expressed in the microenvironment where enkephalins are most active agrees well with the biology of neuropeptide signaling and reinforces our working hypothesis that Aβ may actually work as a neuropeptide important for interneuron function, with proteolysis terminating its activity *in situ*. Furthermore, due to the probable modulation of several neuropeptides by NEP in PV+ synapses, it is possible that elevations in substrates with higher affinity for NEP could negatively impact the levels of other neuropeptides through competitive inhibition.

5. Conclusion

Our results provide a plausible explanation for the compartmentalization of Aβ catabolism and the existence of distinct and independent pools of Aβ in the brain. Analogous to the biology of other neuropeptides, proteolysis of Aβ at inhibitory synapses may be an important mechanism to terminate its activity and curb the spill of Aβ from synapses where Aβ is active. Moreover, with 1.3- and 2-fold increases in endogenous steady-state Aβ observed in ECE-2 and NEP knockout mice, respectively, physiological levels of Aβ within these microenvironments are likely orders of magnitude higher than in the interstitial fluid, potentially reaching concentrations that may facilitate Aβ aggregation.

Exploring whether Aβ has a direct role in modulating inhibitory signaling, and whether alterations in these pools of Aβ can affect synaptic function, Aβ aggregation and neurotoxicity may lead to the identification of more precise and effective therapeutic targets for AD and may help to explain some of the recent failures in drug development for AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Aβ	amyloid-β peptide
AD	Alzheimer’s disease
NEP	neprilysin
ECE	endothelin-converting enzyme

ISH	*in situ* hybridization
IHC	immunohistochemistry
PV	parvalbumin
SOM	somatostatin
GAD67	glutamic acid decarboxylase-67

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Highlights

- Two major Aβ degrading enzymes are selectively expressed by GABAergic interneurons.
- ECE-2 is expressed by somatostatin interneurons and is active in synapses.
- Neprilysin is expressed by parvalbumin interneurons.
- Neprilysin protein localizes to perisomatic parvalbuminergic synapses.

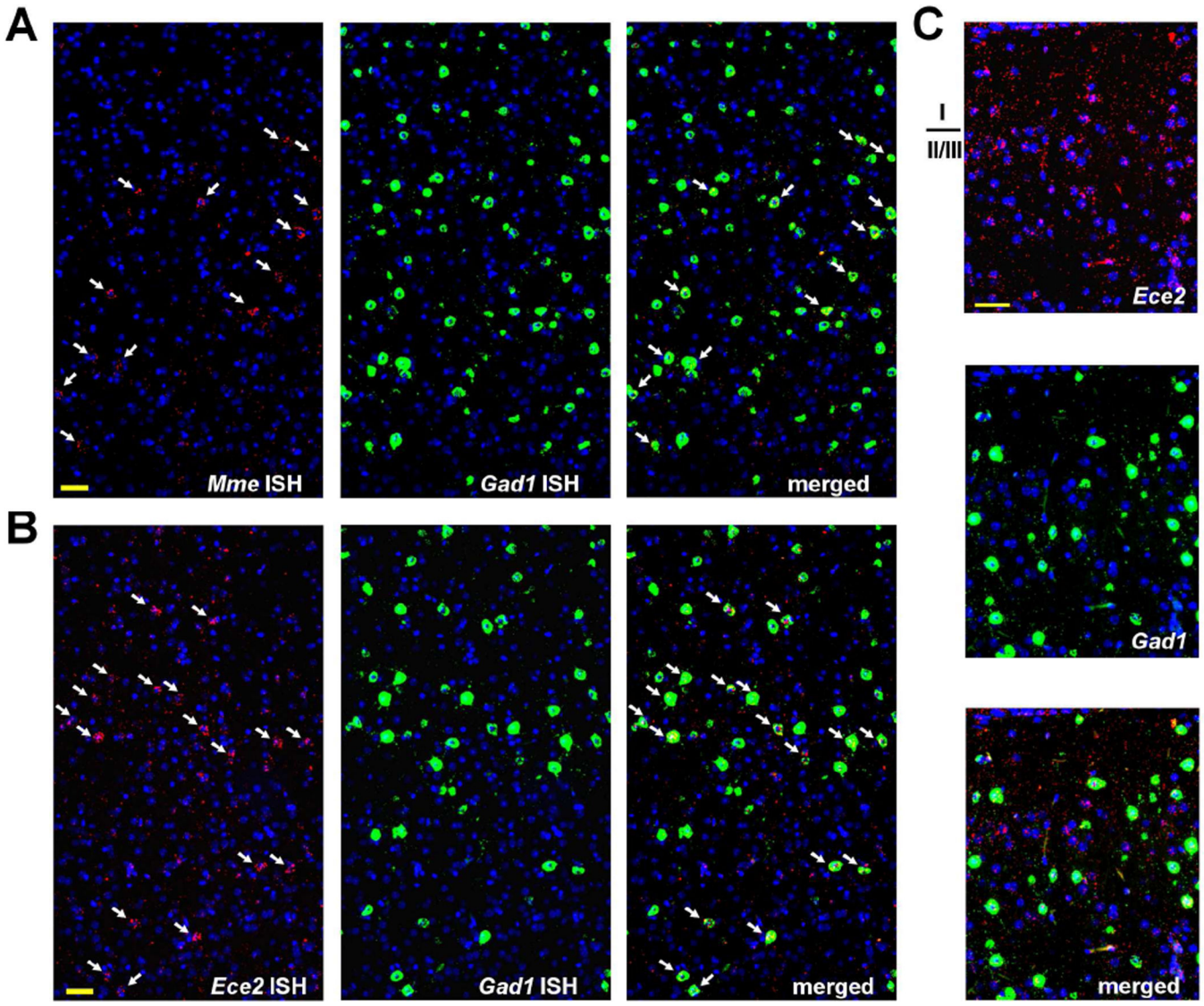

Figure 1. *Ece2* and *Mme* (neprilysin) co-localize with *Gad1* in mouse cortex

As detected by ISH, *Mme* **(A)** and *Ece2* **(B&C)** transcripts, labeled red, were clustered mainly within a small number of cells (arrows) co-expressing the GABAergic marker *Gad1*, labeled green. Nuclei (by Hoechst staining) are labeled blue. Scale bar, 50 μm.

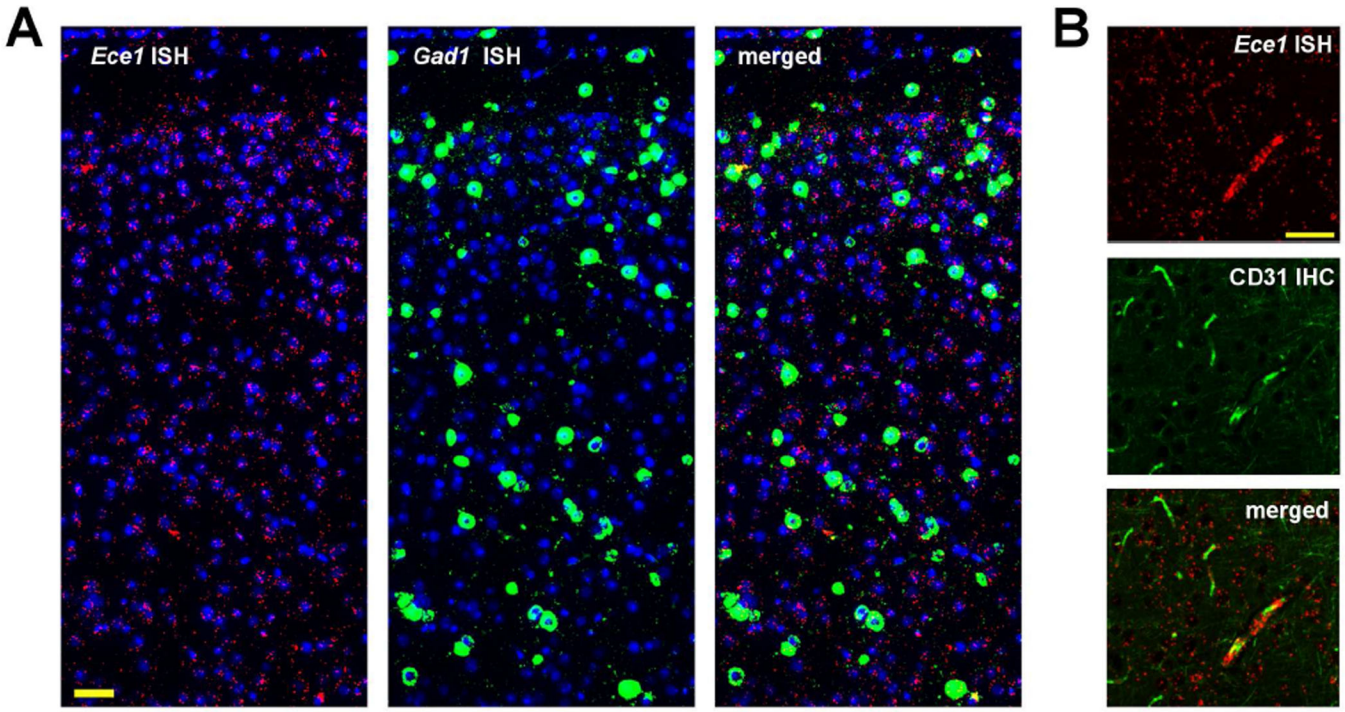

Figure 2. Distribution of *Ece1* mRNA in mouse cortex

(A) *Ece1* transcripts, in red, were only occasionally associated with *Gad1*+ cells, in green. **(B)** Within cerebral blood vessels, *Ece1* transcripts colocalized with PECAM-1/CD-31, detected by IHC and labeled green. Nuclei (by Hoechst staining) are labeled blue. Scale bar, 50 μm.

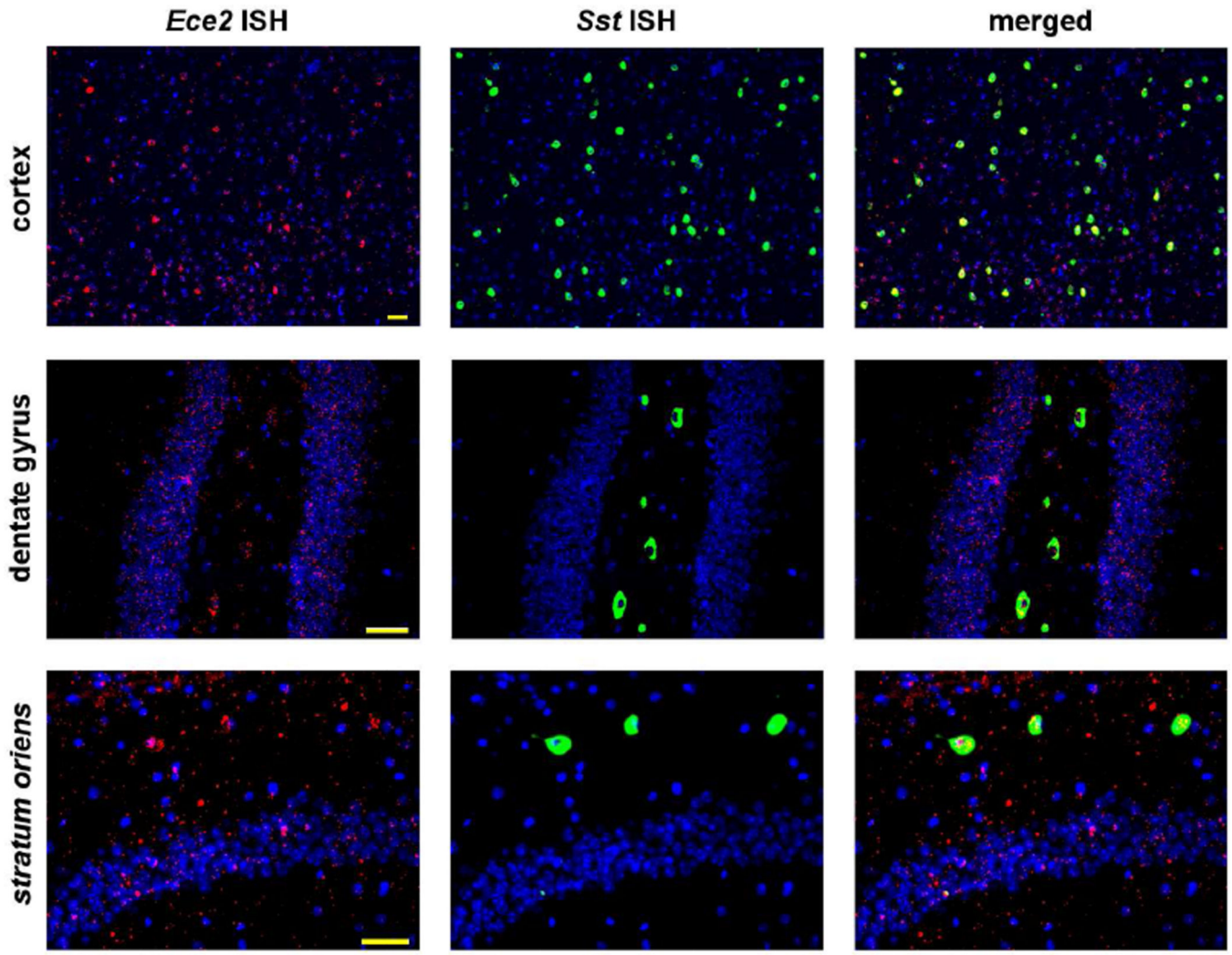

Figure 3. *Ece2* is expressed selectively by somatostatin interneurons

By dual ISH, *Ece2*, labeled red, was detected in the soma of somatostatin (*Sst*)+ cells, labeled green, as well as in a pattern resembling neuronal processes. Nuclei (by Hoechst staining) are labeled blue. Scale bar, 50 μm.

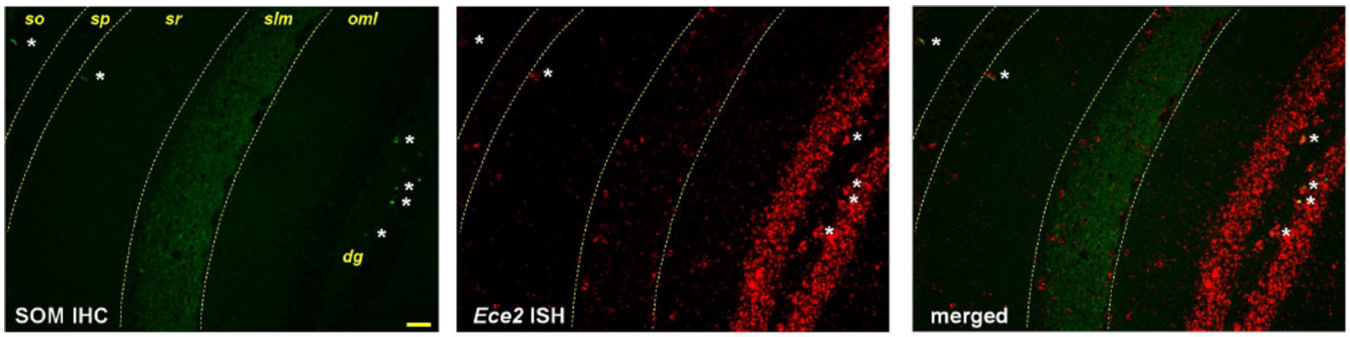

Figure 4. *Ece2* transcripts are found in hippocampal regions where SOM interneurons form synaptic connections

Ece2 mRNA, labeled red, was found in the soma of SOM immunopositive cells (labeled green, white asterisks) as well as in the outer molecular layer (oml) of the dentate gyrus (dg), the *stratum pyramidale* (sp), *stratum radiatum* (sr), and concentrated in the *stratum lacunosum-moleculare* (slm), a highly SOM-immunopositive region. Scale bar, 50 μm.

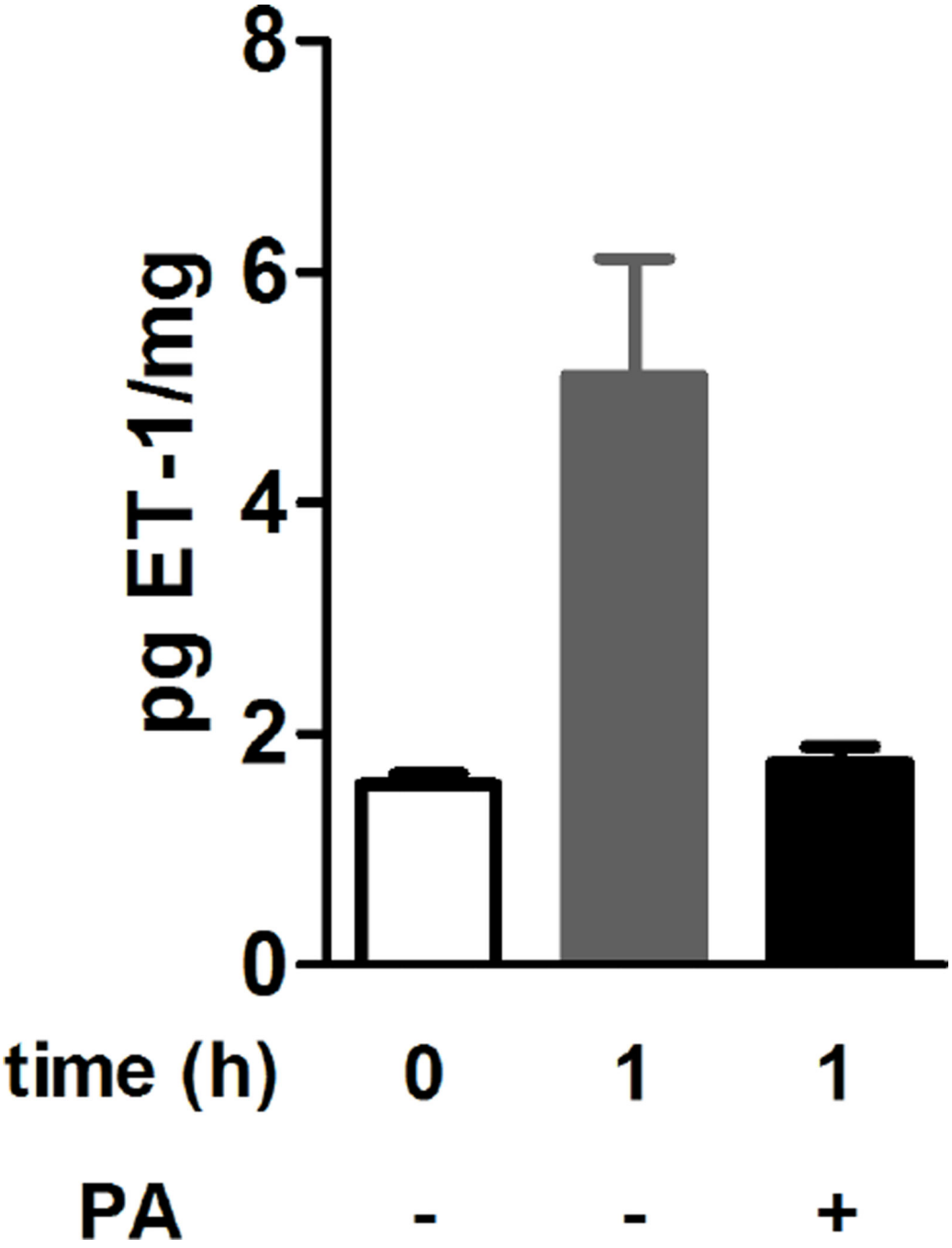

Figure 5. ECE-2 is active in isolated brain synaptosomes

ECE-2 activity was evaluated by measuring the conversion of big ET-1 to ET-1 by isolated brain synaptosomes at pH 5.5, in the presence or absence of the ECE inhibitor phosphoramidon (PA). Zero-time point represents background ET-1 present in synaptosomal preparations. The increase in ET-1 at 1 hr was statistically significant (P=0.0258, unpaired t test). Bars = mean ± SEM of 3 measurements.

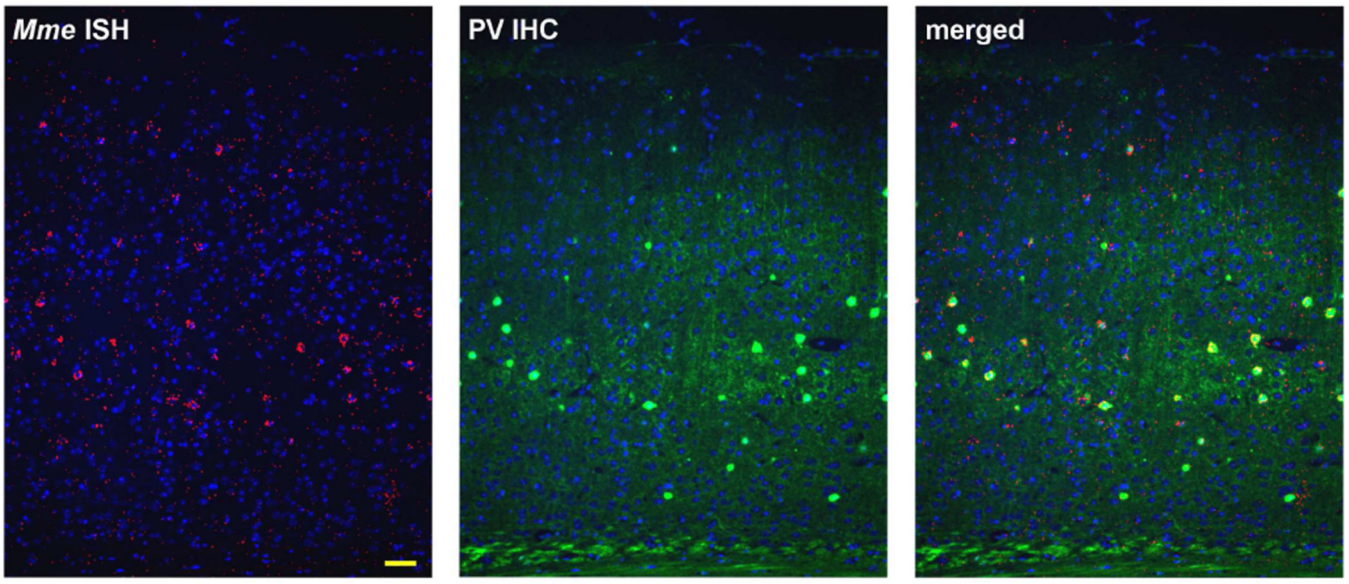

Figure 6. Neprilysin mRNA is enriched in cortical parvalbumin interneurons

Neprilysin (*Mme*) mRNA, in red, was found mostly in the soma of PV immunopositive neurons. Nuclei (by Hoechst staining) are labeled blue. Scale bar, 50 μm.

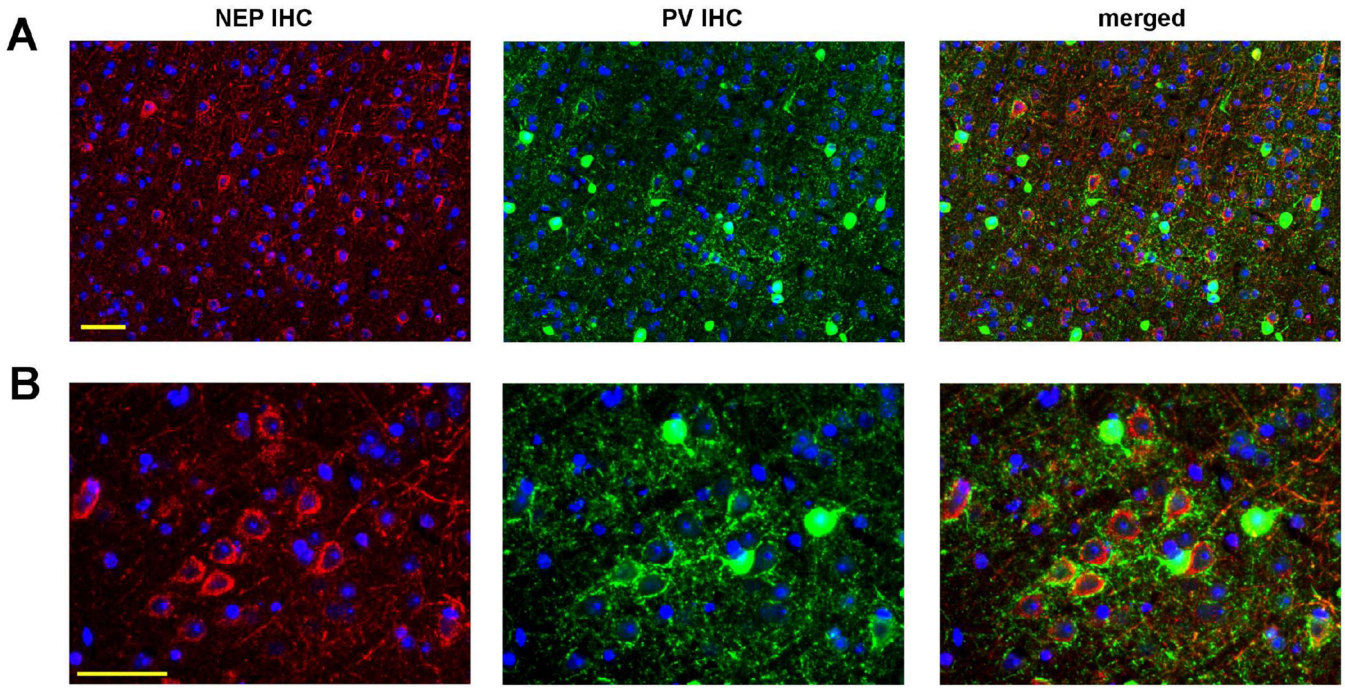

Figure 7. Neprilysin protein localizes to the presynaptic endings of cortical parvalbumin interneurons

(A) By double immunofluorescence, NEP protein was found enriched at the end terminals of PV+ neurons surrounding the soma of principal cells. The area shown corresponds to layer 4/5 of somatosensory cortex. **(B)** Higher magnification image of a similar area. Nuclei (by Hoechst staining) are labeled blue. Scale bar, 50 μm.

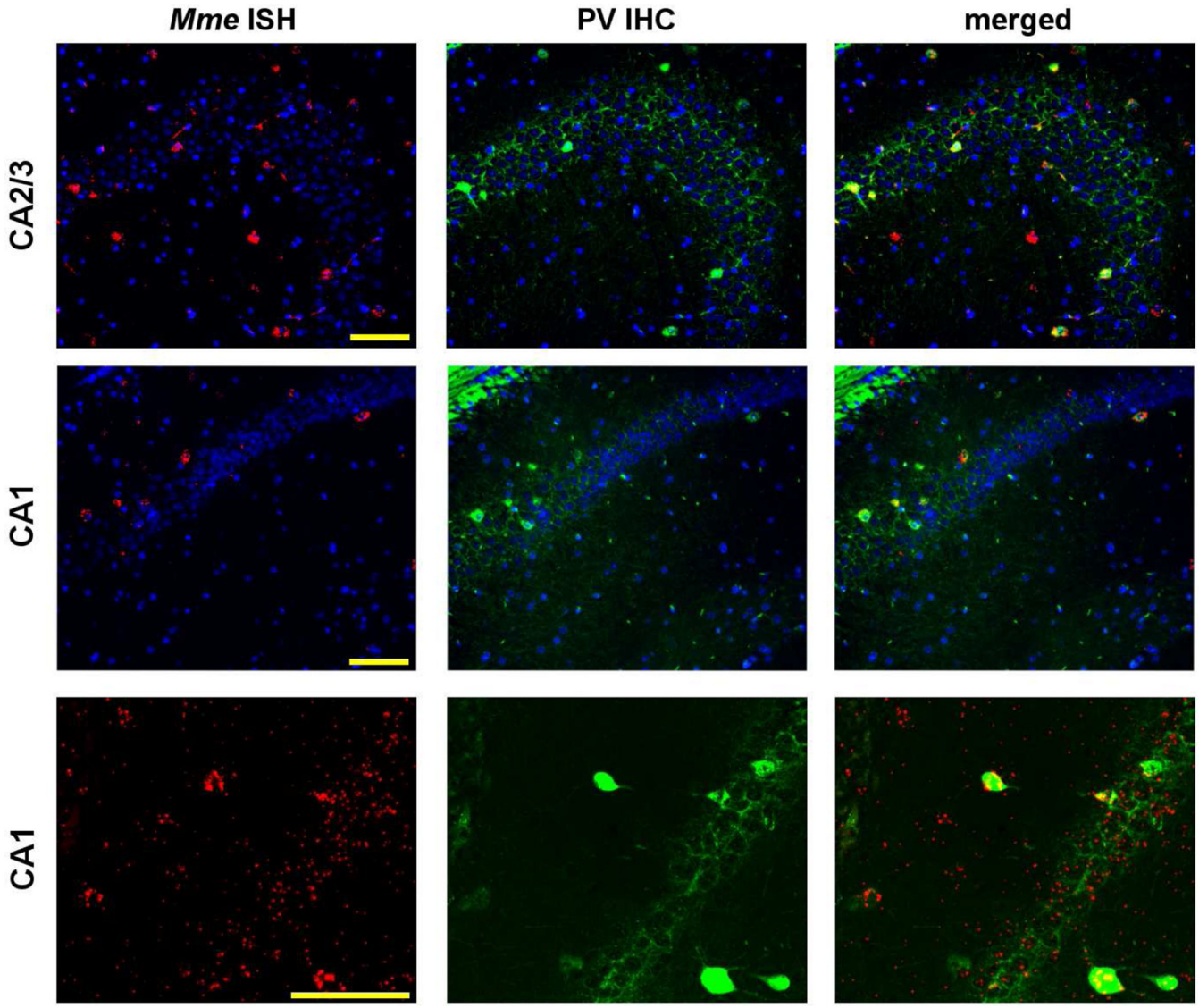

Figure 8. *Mme* expression in hippocampus

Mme+ cells (in red) were found adjacent to the pyramidal layer of the CA1 and CA2/3 and the majority were immunoreactive for PV (in green). At higher magnification (bottom panels), *Mme* mRNA could be observed in the soma of PV+ cells as well as in PV+ processes surrounding the CA1 pyramidal cell layer. Nuclei (by Hoechst staining) are labeled blue. Scale bar, 50 μm.

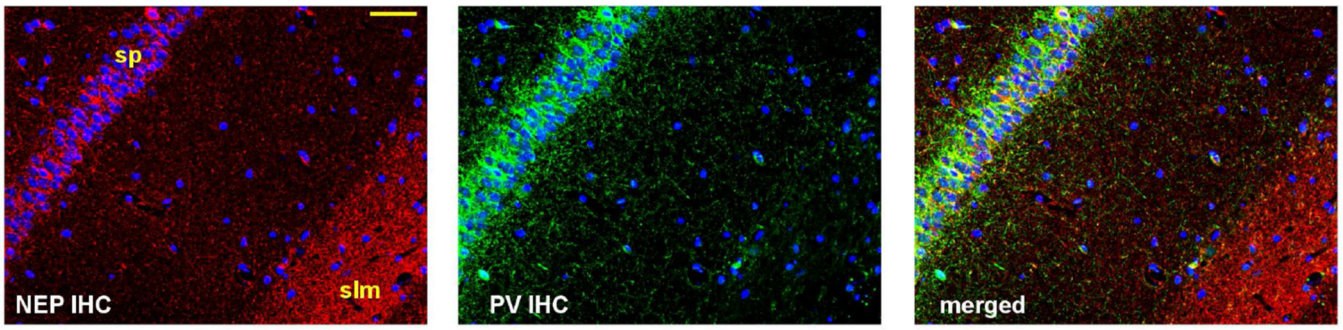

Figure 9. NEP protein localization in hippocampus

NEP immunostaining (in red) was detected in PV immunopositive fibers (in green) in the *stratum pyramidale* (sp) and was highly enriched in *stratum lacunosum moleculare* (slm). Nuclei (by Hoechst staining) are labeled blue. Scale bar, 50 μm.

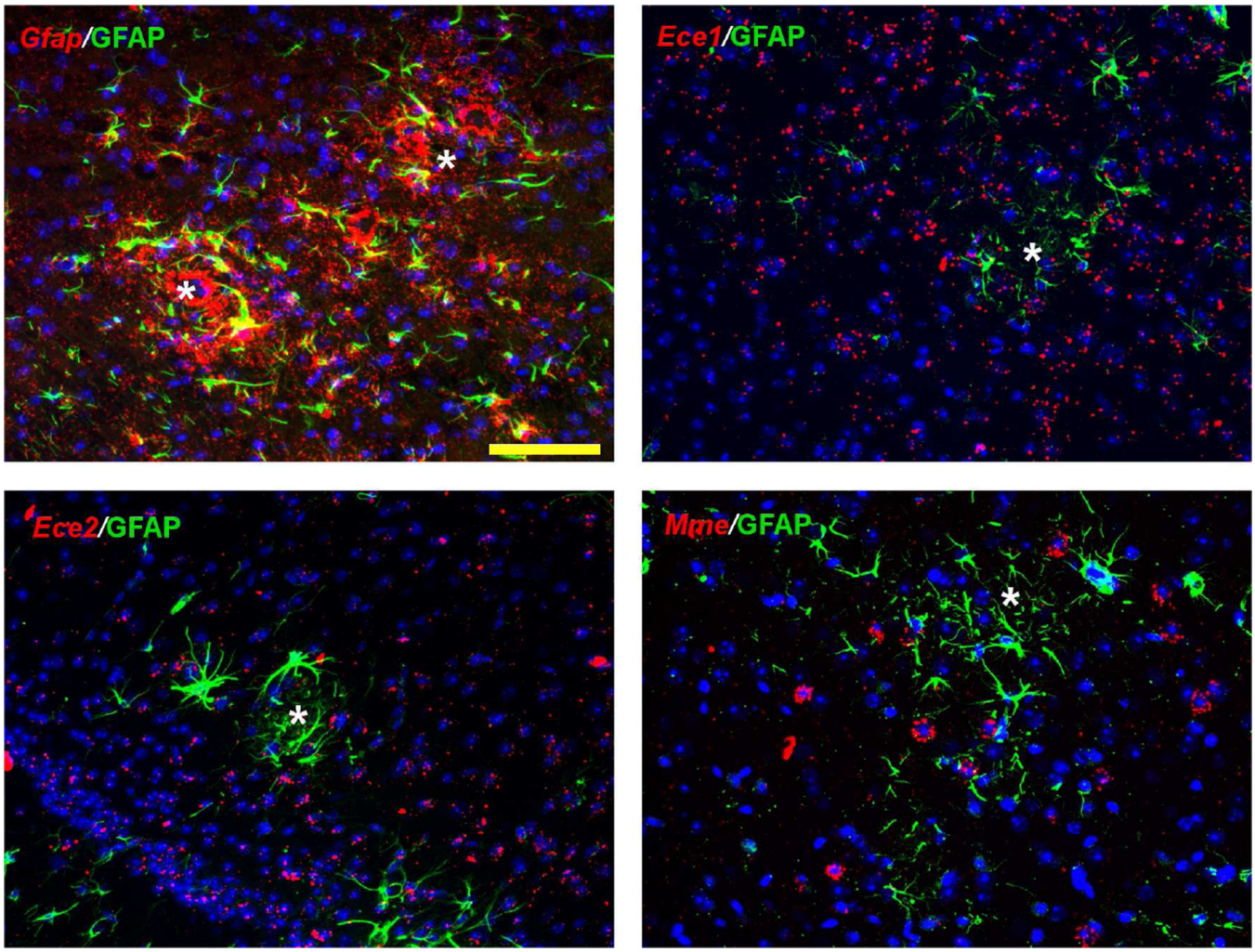

Figure 10. Expression of Aβ degrading enzymes in peri-plaque areas with gliosis

In brains of TgCRND8 APP transgenic mice, *Gfap* mRNA (red, upper left panel) was prominent surrounding amyloid plaques (white asterisks) and coincided with increased GFAP immunoreactivity (labeled green in all panels). Expression of *Ece1, Ece2*, and *Mme* (in red) was not apparently increased compared to surrounding areas. Nuclei (by Hoechst staining) are labeled blue. Scale bar, 50 μm.