Metabolic Regulation of Brain Aβ by Neprilysin

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Amyloid β peptide (Aβ), the pathogenic agent of Alzheimer's disease (AD), is a physiological metabolite in the brain. We examined the role of neprilysin, a candidate Aβ-degrading peptidase, in the metabolism using neprilysin gene-disrupted mice. Neprilysin deficiency resulted in defects both in the degradation of exogenously administered Aβ and in the metabolic suppression of the endogenous Aβ levels in a gene dose-dependent manner. The regional levels of Aβ in the neprilysin-deficient mouse brain were in the distinct order of hippocampus, cortex, thalamus/striatum, and cerebellum, where hippocampus has the highest level and cerebellum the lowest, correlating with the vulnerability to Aβ deposition in brains of humans with AD. Our observations suggest that even partial down-regulation of neprilysin activity, which could be caused by aging, can contribute to AD development by promoting Aβ accumulation.

The decades-long pathological cascade of Alzheimer's disease (AD) is initiated by the deposition of Aβ in the brain (*1*, *2*). Aβ is a physiological peptide, the steady-state levels of which are determined by the balance between the anabolic and catabolic activities (*3*, *4*). Because an increase of only 50% in the production of a particular form of Aβ, caused by the majority of familial AD mutations, leads to aggressive presenile Aβ pathology (*1*, *2*), subtle alterations in this metabolic balance over a long period of time are likely to influence not only the pathological progression but also the incidence of the disease. We have focused our attention on the catabolism rather than anabolism of Aβ because the former is much less well understood and because reduced catabolism may, by promoting Aβ deposition, account for the unresolved mechanism of late-onset AD development (*5*).

We previously demonstrated that a neutral endopeptidase sensitive to both phosphoramidon and thiorphan plays a major rate-limiting role in $Aβ_{1-42}$ catabolism (*6*). The exact molecular identity of the endopeptidase, however, has remained unclear because at least six endopeptidases with similar biochemical properties are known to be present in brain (*7*–*9*). Despite this apparent molecular redundancy for the endopeptidase activity, neprilysin appears to be the major Aβ-degrading enzyme because this peptidase accounts for the majority of the inhibitor-sensitive endopeptidase activity in the brain and exhibits the most potent Aβ-degrading activity among these isozymes (*9*, *10*). Therefore, we hypothesized that if neprilysin is indeed the rate-limiting enzyme, then neprilysin deficiency would influence the steady-state Aβ levels in the brain by altering the metabolism.

We first analyzed the in vivo degradation of exogenously administered Aβ in neprilysin gene-disrupted mice. The mice are known to be normal in reproductive, developmental, and physiological aspects (*11*), presumably due to the redundancy of the enzyme activity, and thus are suitable for post-developmental studies. Figure 1 shows the degradation profile of $^{3}H/^{14}C$-labeled human-type $Aβ_{1-42}$ peptide injected into mouse brains. The peptide and catabolites were extracted 30 min after the injection and subsequently were analyzed by high-performance liquid chromatography (HPLC) in connection with a flow scintillation analyzer (*6*). The peaks at the retention times of 7 to 8 and 42 min correspond to catabolites and the intact Aβ peptide, respectively. The $Aβ_{1-42}$ peptide underwent 70 to 80% degradation in 30 min and the degradation was effectively blocked by a neprilysin inhibitor, thiorphan. In contrast, the majority of the $Aβ_{1-42}$ peptide injected into the neprilysin-deficient (–/–) mouse brain remained unmetabolized even in the absence of thiorphan. Aβ degradation was also decelerated, though to a lesser extent, in the heterozygously deficient (+/–) mice, consistent with the notion that the peptidase action is a rate-limiting process (*6*).

The data also indicate the presence of alternative or minor neprilysin-independent catabolic pathway(s) because a small fraction of injected Aβ still underwent degradation in the neprilysin-deficient mice (arrowhead, Fig. 1B). A thiorphan-sensitive neprilysin homolog, neprilysin-like peptidase α, may account for such activity because this isozyme is the only neprilysin family member capable of degrading Aβ in a manner almost comparable to that of neprilysin (*9*). Other candidates include insulin-degrading enzyme and plasmin (*12*–*14*). We did not observe pathological Aβ deposition in the mice null solely of neprilysin, presumably due to this molecular redundancy.

If neprilysin is the major Aβ-degrading enzyme in vivo, neprilysin deficiency should also result in the elevation of endogenous Aβ levels in the brain. For quantitative analysis, we used an enzyme-linked immunosorbent assay (ELISA) identical to the one used to examine the effect of presenilin mutations in transgenic mice (*15*–*17*). This is the only ELISA that has been established as sensitive

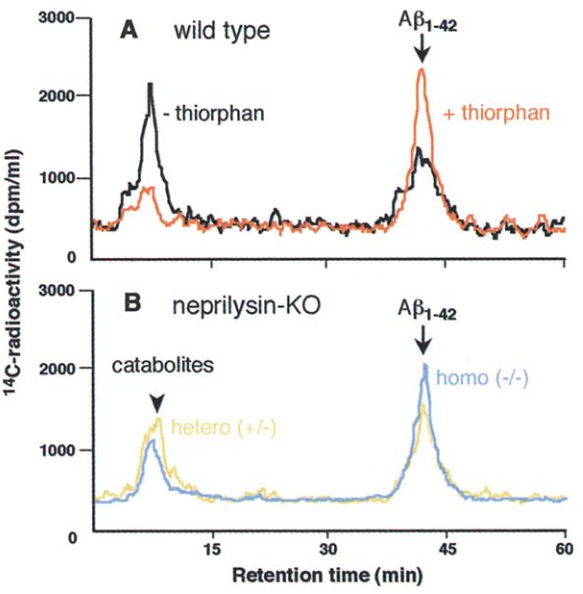

Fig. 1. Degradation of $^{3}H/^{14}C$-labeled $Aβ_{1-42}$ peptide in wild-type and neprilysin-deficient mouse brains. The radiolabeled peptide was injected into stereotaxically measured location of the hippocampus of 8-week-old neprilysin$^{+/+}$ mice in the absence (black) or presence (red) of thiorphan (**A**) and of neprilysin$^{-/-}$ (blue) and neprilysin$^{+/-}$ (yellow) mice (**B**) and subsequently was analyzed as previously described (*6*), except that 0.2 μl of 0.2 μg $^{3}H/^{14}C$-$Aβ_{1-42}$ solution per animal was used. Thiorphan (1 mM) was injected simultaneously with the Aβ peptide. Each result shows the sum of three independent injection experiments. Each set of experiments was repeated at least two times to confirm the results. KO, knock-out; dpm, disintegrations per minute.

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and specific enough for quantifying endogenous $A\beta_{40}$ and $A\beta_{42}$ (Aβ ending at residues 40 and 42, respectively) from mouse brain. The levels of $A\beta_{40}$ and $A\beta_{42}$ were significantly elevated in the neprilysin-deficient mice in a gene dose-dependent manner (Table 1). For a positive control, we analyzed mice carrying a familial AD-causing presenilin 1 gene mutation (*18*) and confirmed selective 1.5-fold increase in $A\beta_{42}$ content as previously established (*1*, *2*, *15–18*). Thus, the increase in $A\beta_{42}$ caused by the heterozygous neprilysin deficiency is comparable to that caused by presenilin mutations (*15–18*). In contrast, the enkephalin levels do not increase in the neprilysin-deficient mice (*19*), despite the fact that neprilysin is a potent enkephalin degrader previously termed "enkephalinase," indicating that neprilysin deficiency is compensated for by other mechanisms in the case of enkephalin metabolism.

Most important, the inverse correlation between the neprilysin gene dose and Aβ levels (Table 1) suggests that even a partial down-regulation of neprilysin activity, which could be caused by aging, will promote Aβ deposition in the brain. This finding agrees with a recent report describing the selective reduction of neprilysin expression in brains with sporadic AD, particularly in high plaque regions such as hippocampus and temporal gyrus (*20*). Because the expression of two controls, cyclophilin and microtubule-associated protein 2, is not reduced in AD, down-regulation of neprilysin does not seem to be a simple consequence of neurodegeneration. If this change precedes the disease onset, neprilysin down-regulation could be causal to the Aβ deposition in these AD cases.

Because one feature of Aβ pathology in AD brain is the distinct regional difference in severity (*21*), we quantified the Aβ levels in various brain regions in wild-type and neprilysin-deficient mice (Fig. 2). The Aβ levels in the neprilysin-deficient mice were in the order of hippocampus, cortex, thalamus/striatum, and cerebellum (from highest to lowest, respectively), which correlates well with the severity of Aβ pathology in AD brains (*20*, *21*). The minimal relative increase of $A\beta_{40}$ in the cerebellum, a region in which mature cored Aβ plaques containing $A\beta_{40}$ are rarely observed in humans (*22*), is also consistent.

The neprilysin gene-dosage effect on the brain Aβ levels (Table 1) allows us to speculate on its possible medical application. Because the wild-type neprilysin$^{+/+}$ mice can be regarded as transgenic for neprilysin as compared to the heterozygous neprilysin$^{+/-}$ mice, the difference in the Aβ levels observed between the +/+ and +/– mice suggests that elevating neprilysin activity in brains containing high Aβ levels by gene therapy or transcriptional up-regulation, for instance (see below), is likely to effectively lower Aβ levels. This strategy of catabolic up-regulation would be complementary to that of anabolic down-regulation, i.e., secretase inhibition, without side effects on the processing of amyloid precursor protein (APP) and other secretase substrates (*5*). These two strategies may be combined together to achieve a maximum effect.

Lastly, our observations may provide new insights for the genetics of AD. As shown in Fig. 3A, expression of the neprilysin gene is transcriptionally regulated in a tissue-specific manner (*23*, *24*). The major mRNA transcripts with distinct tissue distributions vary in their 5′ noncoding regions. Because the predominant form expressed in neurons is the type 1 tran-

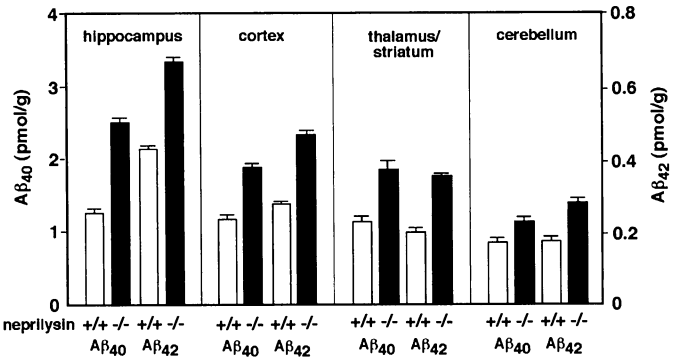

Fig. 2. $A\beta_{40}$ and $A\beta_{42}$ in four brain regions of wild-type and neprilysin-deficient mice. Aβ from the hippocampus, cortex, thalamus/striatum, and cerebellum regions of mouse brains was extracted and quantified as described in Table 1. Each bar represents the average ± SE (n = 4 mouse brains).

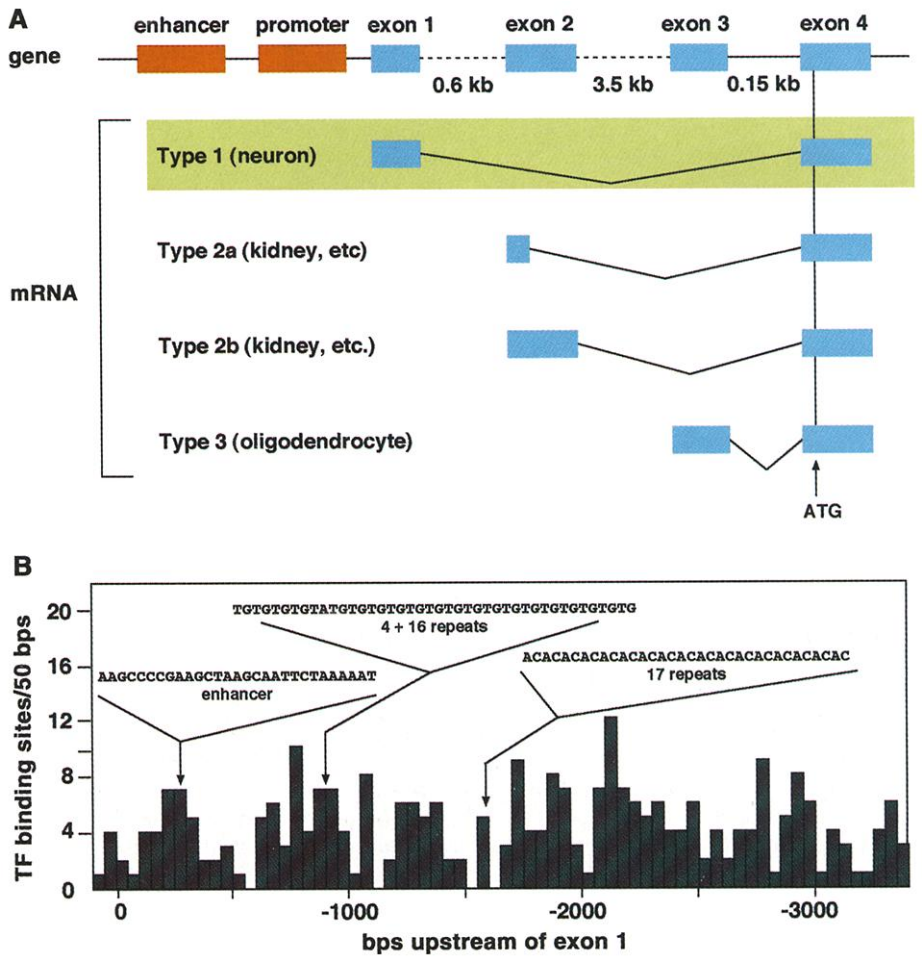

Fig. 3. Biology-based prediction of genetic risk factor(s). (**A**) Schematic representation of the neprilysin gene and mRNA transcripts. Four types of neprilysin mRNA transcripts are generated through the ligation of different noncoding exons to the common coding exon and alternative splicing. Type 1 is the predominant form expressed in neurons. (**B**) Transcription factor (TF) binding sites, an enhancer sequence, and dinucleotide repeats in the human genome region up-stream of exon 1. The human sequence homologous to the rat enhancer sequence is shown. The number of TF binding sites was assessed using an algorithm for the search of binding site candidates, available at www.etl.go.jp/etl/cbrc/research/db/TFSEARCH.html. bps, base pairs.

Table 1. $A\beta_{40}$ and $A\beta_{42}$ levels in wild-type and neprilysin-deficient mice. $A\beta_{40}$ and $A\beta_{42(43)}$ were extracted from mouse brains by guanidine hydrochloride and quantified as described (*6*). The antibodies for the ELISA were generously provided by Takeda Chemical Industries, Ltd. Eight-week-old male mice were used for all experiments. We performed eight independent measurements to examine the effect of neprilysin deficiency using more than 50 mice; all the results were consistent. For neprilysin, $n = 9$ mouse brains; for presenilin, $n = 3$ mouse brains. The amounts of APP and its proteolytic fragments remained unchanged in neprilysin$^{-/-}$ mice as analyzed by Western blotting (*30*). The positive control data were taken in an identical manner using mutant presenilin-1 knock-in mice (*18*). Each value represents the average ± SE with the indicated number of animals.

Genotype	$A\beta_{40}$ (pmol/g)	$A\beta_{42}$ (pmol/g)
	Neprilysin	
+/+ (control littermates)	1.084 ± 0.075	0.253 ± 0.011
+/−	1.475 ± 0.047*	0.398 ± 0.045*
−/−	2.174 ± 0.130*	0.541 ± 0.063*
	Mutant presenilin 1	
−/− (control littermates)	1.047 ± 0.138	0.252 ± 0.036
−/+	1.176 ± 0.183	0.368 ± 0.029†

*$P < 0.001$ and †$P < 0.01$, as compared to control littermates by Student's t test.

script containing exon 1, whereas the other forms are the major transcripts found in other tissues, the enhancer and promoter regions upstream of exon 1 (*23–25*) are likely to selectively regulate the total expression level of neprilysin in neurons. Indeed, there are several clusters of possible transcription factor (TF) binding sites, at least one identified enhancer, and two dinucleotide repeats in the upstream region (Fig. 3B). Removal of the enhancer sequence leads to more than 90% reduction in promoter activity (*25*). The neprilysin gene also possesses two androgen-responsive elements (*26*), which might be associated with the lower incidence of the disease among males than females (*27*). Therefore, it may be possible that some of the mutations or polymorphisms in these and related regions could influence the expression of neprilysin in a neuron-specific manner and consequently alter Aβ levels in the brain. Such mutations or polymorphisms can be either a risk factor or protective factor, depending on whether they cause down- or up-regulation of neprilysin expression. Although this assumption is a hypothetical prediction, the neprilysin gene is indeed located within the candidate chromosome 3 locus associated with late-onset AD cases (*28*, *29*) and is, therefore, a potential target in the search for genetic risk factors.

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Impairment of the Ubiquitin-Proteasome System by Protein Aggregation

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Intracellular deposition of aggregated and ubiquitylated proteins is a prominent cytopathological feature of most neurodegenerative disorders. Whether protein aggregates themselves are pathogenic or are the consequence of an underlying molecular lesion is unclear. Here, we report that protein aggregation directly impaired the function of the ubiquitin-proteasome system. Transient expression of two unrelated aggregation-prone proteins, a huntingtin fragment containing a pathogenic polyglutamine repeat and a folding mutant of cystic fibrosis transmembrane conductance regulator, caused nearly complete inhibition of the ubiquitin-proteasome system. Because of the central role of ubiquitin-dependent proteolysis in regulating fundamental cellular events such as cell division and apoptosis, our data suggest a potential mechanism linking protein aggregation to cellular disregulation and cell death.

The ubiquitin-proteasome system (UPS) functions in cellular quality control by degrading misfolded, unassembled, or damaged proteins that could otherwise form potentially toxic aggregates (*1*). Because multiubiquitylated proteins are usually efficiently degraded by cellular proteasomes, the presence of elevated ubiquitin conjugates associated with intracellular deposits of aggregated protein in diseased neurons in nearly all sporadic and hereditary neurodegenerative diseases has long suggested a linkage between UPS dysfunction and pathogenesis (*2*). Recently this linkage has been strengthened by genetic evidence linking mutations in the UPS to several neurodegenerative diseases and models thereof (*3–7*). Despite this evidence, however, the specific causal relation between protein aggregation, UPS activity, and pathogenesis has remained elusive.

To investigate the specific relation between protein aggregation and the function of the UPS, we designed a reporter consisting of a short degron, CL1 (*8*), fused to the COOH-terminus of green fluorescent protein (GFP^u) (*9*). A clonal line of human embryonic kidney (HEK) 293 cells stably expressing GFP^u was isolated and designated GFP^u-1. Pulse-chase analysis (*10*) (Fig. 1A) indicated that GFP^u is

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