Loss of kallikrein-related peptidase 7 exacerbates amyloid pathology in Alzheimer’s disease model mice

Kiwami Kidana1,2,3,†, Takuya Tatebe1,†, Kaori Ito4, Norikazu Hara5, Akiyoshi Kakita6, Takashi Saito7, Sho Takatori1, Yasuyoshi Ouchi2,8, Takeshi Ikeuchi5, Mitsuhiro Makino4, Takaomi C Saido7,8, Masahiro Akishita2, Takeshi Iwatsubo9, Yukiko Hori1 & Taisuke Tomita1,*,†

Abstract

Deposition of amyloid-β (Aβ) as senile plaques is one of the pathological hallmarks in the brains of Alzheimer’s disease (AD) patients. In addition, glial activation has been found in AD brains, although the precise pathological role of astrocytes remains unclear. Here, we identified kallikrein-related peptidase 7 (KLK7) as an astrocyte-derived Aβ degrading enzyme. Expression of KLK7 mRNA was significantly decreased in the brains of AD patients. Ablation of Klk7 exacerbated the thioflavin S-positive Aβ pathology in AD model mice. The expression of Klk7 was upregulated by Aβ treatment in the primary astrocyte, suggesting that Klk7 is homeostatically regulated by Aβ-induced responses. Finally, we found that the Food and Drug Administration-approved anti-dementia drug memantine can increase the expression of Klk7 and Aβ degradation activity specifically in the astrocytes. These data suggest that KLK7 is an important enzyme in the degradation and clearance of deposited Aβ species by astrocytes involved in the pathogenesis of AD.

Keywords Alzheimer’s disease; amyloid-β; astrocyte; kallikrein-related peptidase 7; protease

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Introduction

Alzheimer’s disease (AD) is the most common type of dementia. Genetic and biochemical evidence suggests that the aggregation and deposition of amyloid-β (Aβ) are critical processes in the pathogenesis of AD (Holtzman et al., 2011). Aβ is produced upon proteolysis of amyloid precursor protein (APP). Several genetic mutations linked to familial AD increase the production or aggregation of Aβ. In contrast, sporadic AD patients have been reported to have a decreased clearance rate, rather than an increased production rate of brain Aβ (Mawuenyega et al., 2010). Thus, understanding the molecular mechanism of brain “Aβ economy” (Karran et al., 2011), which reflects the balance of the rates of production, clearance, and aggregation of Aβ, is crucial for the development of effective therapeutics for AD. However, the whole picture of the pathophysiological Aβ clearance/degradation pathway in the brain is still unclear (Saido & Leissring, 2012). To date, several enzymes, including neprilysin (membrane metallo-endopeptidase) and insulin-degrading enzyme, have been identified as Aβ-degrading enzymes (Nalivaeva et al., 2014). Among them, the knockout of neprilysin or insulin-degrading enzyme in mice showed an approximately twofold increase in the level of soluble brain Aβ, although the effect on Aβ deposition in vivo remains controversial (Iwata et al., 2000, 2001; Farris et al., 2003; Leissring et al., 2003). Importantly, in a therapeutic context, remaining neurons might not be a suitable target to remove Aβ deposits in the treatment for AD. Thus, we focused on astrocytes, which play important roles in physiological and pathological functions in the brain (De Strooper & Karran, 2016; Pekny et al., 2016). Of note, changes in the phenotypes of astrocytes, but in not their number, were associated with the clinical pathology of AD.
(Serrano-Pozo et al, 2013). However, the precise mechanism of astrocyte-mediated Aβ clearance remains unclear.

Fifteen kallikrein-related peptidase (KLK) family proteins have been identified and act in a complex network as a cascade reaction. Among them, KLK6 (neurosin) and KLK8 (neuropsin) are major kallikrein-related peptidase proteins in the central nervous system (Sotiropoulou et al, 2009; Prassas et al, 2015). KLK6 is widely expressed in several cells, and KLK8 is expressed in neurons. Also, KLK8 has been implicated in the pathogenesis of AD (Herring et al, 2016). KLK7 was originally identified as an inflammation-induced proteolytic enzyme in the skin. However, the expression level of KLK7 was decreased in the cerebrospinal fluid and brain of AD patients (Diamandis et al, 2004; Bossers et al, 2010). Moreover, it was reported that KLK7 is capable to cleave the hydrophobic core motif of Aβ fibrils, thereby attenuating neurotoxicity in vitro (Shropshire et al, 2014). In this manuscript, we have investigated the pathophysiological impact of KLK7 in brain Aβ economy and identified KLK7 as the astrocyte-derived Aβ-degrading enzyme that regulates amyloid pathology in vivo.

Results

Identification of KLK7 as the Aβ-degrading enzyme

To identify the proteolytic enzyme that is capable to degrade secreted Aβ species, we utilized the conditioned medium from 7PA2 cells as a substrate source, as 7PA2 cells secrete toxic human Aβ oligomer species (Podlisny et al, 1995). We took the conditioned medium from several cell lines and mixed with that from 7PA2 cells. The mixture was separated by Urea-containing SDS–PAGE gel, which enables us to discriminate different C-terminal length of Aβ immunoblot (Klafki et al, 1996; Qi-Takahara et al, 2005). We found that the media from astrocytoma CCF-STTG1, neuroglioma H4, neuroblastoma SH-SYSY, and glioblastoma U87 cells showed robust Aβ-degrading activity in this condition (Fig 1A, and Appendix Fig S1A and B). In addition, the conditioned medium of CCF-STTG1 cells also showed the degrading activity on Aβ derived from human neuroblastoma BE(2)-C cells (Appendix Fig S1C). We then further characterized the degradation activity using CCF-STTG1 cells that showed strongest activity. This activity was specifically inhibited by diisopropyl fluorophosphates as well as tosyl phenylalanyl chloromethyl ketone (Fig 1B and C), indicating that KLK7 showed the degradation activity against naturally secreted Aβ (Fig 3C and Appendix Fig S3B), which was abolished by the addition of MAB2624. Finally, coinubcation of recombinant purified human KLK7, but not KLK6, derived from either mamalian cells or bacteria with 7PA2-derived Aβ or the synthetic Aβ resulted in a significant Aβ degradation in vitro (Fig 3D and Appendix Fig S3C–E), indicating that KLK7 is directly involved in the Aβ degradation. Consistent with previous result (Shropshire et al, 2014), KLK7 degraded synthetic Aβ fibrils (Fig 3E). To further elucidate the role of KLK7 in murine astrocyte, we analyzed the primary glial cells obtained from rat pups. This culture is mainly comprised of primary astrocytes, but still contained the primary microglia. Notably, Klk7-positive puncta were detected only in Aldh1L1-positive primary astrocytes, but not in Iba-1-positive microglia (Appendix Fig S4A). Conditioned medium from the primary glial cell culture also showed chymotrypsin-type serine protease-dependent Aβ degradation activity inhibited by the neutralizing antibody against KLK7 (Fig 4A and B, and Appendix Fig S4B). Other proteases might be also involved in the Aβ degradation because the MAB2624 showed partial inhibition in this assay. However, these results implicated that Klk7-dependent Aβ degradation activity was associated with the astrocytes.

Importance of KLK7 in the brain Aβ pathology

We then generated homozygous Klk7 knockout mice (Klk7^−/−) using ES cells carrying Klk7^tm1(KOMP)Vlcg VelociGene deletion allele. Klk7^−/− mice appeared normal and were fertile, and their expression of Klk7 mRNA was completely abolished (Appendix Fig S5A–C). Moreover, MAB2624 failed to inhibit the Aβ degradation in the conditioned medium from the primary glial culture obtained from Klk7^−/− mice, indicating that MAB2624 specifically inhibited the proteolytic activity of KLK7 protein in the degradation assay (Appendix Fig S5D). However, no commercial antibody detected the endogenous brain KLK7 protein on the immunoblot. Then, we analyzed the levels of endogenous murine brain Aβ and found 1.4-fold to twofold increase in both male and female Klk7^−/− mouse brains (Appendix Fig S5E). No change was observed in the expression levels of APP, proteolytic fragments of APP, ADAM10, BACE1,
KLK7 is physiologically involved in the catabolism of brain Aβ. Treatment with recombinant KLK7 protein into hippocampi of wild-type mouse significantly decreased the immunoreactivity in the sandwich enzyme-linked immunosorbent assay (Saito et al., 2001) and decreases the immunoreactivity in the sandwich enzyme-linked immunosorbent assay (Saito et al., 2014, 2016) under a Klk7-null background. Homozygous AppNL-G-F/NL-G-F mice showed cortical amyloid deposition at 1–2 months of age, and thioflavin S-positive plaques that were surrounded by glial fibrillary acidic protein (GFAP)-positive astrocytes appeared at 6–7 months of age. As the Arctic mutation in the knockin allele promotes aggregation (Nilserth et al., 2001) and decreases the immunoreactivity in the sandwich enzyme-linked immunosorbent assay (Saito et al., 2014), we compared the brain Aβ levels by immunoblot (Fig 5A–C and Appendix Fig S6A–C). We observed a significant increase in the levels of Tris buffer-soluble and SDS-soluble and formic acid-soluble) were also increased. Consistent with these biochemical analyses, brain amyloid deposition was drastically increased (5.6-fold) in the brains of AppNL-G-F/NL-G-F; Klk7 mice (Fig 5D and E). These data implicated a significant impact of loss of Klk7 ablation without affecting APP and related proteins (Appendix Fig S7A). Moreover, amounts of insoluble Aβ (i.e., SDS-soluble and formic acid-soluble) were also increased. Consistent with these biochemical analyses, brain amyloid deposition was drastically increased (5.6-fold) in the brains of AppNL-G-F/NL-G-F; Klk7 mice (Fig 5D and E). These data implicated a significant impact of loss of Klk7 not only on the biochemical Aβ economy, but on the deposition pattern of the Aβ plaques.

Then, we examined the amyloid pathology of App knockin mice (Saito et al., 2014, 2016) under a Klk7-null background. Homozygous AppNL-G-F/NL-G-F mice showed cortical amyloid deposition at 1–2 months of age, and thioflavin S-positive plaques that were surrounded by glial fibrillary acidic protein (GFAP)-positive astrocytes appeared at 6–7 months of age. As the Arctic mutation in the knockin allele promotes aggregation (Nilserth et al., 2001) and decreases the immunoreactivity in the sandwich enzyme-linked immunosorbent assay (Saito et al., 2014), we compared the brain Aβ levels by immunoblot (Fig 5A–C and Appendix Fig S6A–C). We observed a significant increase in the levels of Tris buffer-soluble and SDS-soluble and formic acid-soluble) were also increased. Consistent with these biochemical analyses, brain amyloid deposition was drastically increased (5.6-fold) in the brains of AppNL-G-F/NL-G-F; Klk7 mice (Fig 5D and E). These data implicated a significant impact of loss of Klk7 not only on the biochemical Aβ economy, but on the deposition pattern of the Aβ plaques.

We further analyzed the pathological changes related to the Aβ deposition. We observed the accelerated phosphorylation of murine endogenous tau (Appendix Fig S6A and B) as well as formation of BACE1-accumulated dystrophic neurites (Appendix Fig S8C) (Kandalepas et al., 2013) in the brains of AppNL-G-F/NL-G-F; Klk7 mice. In addition, increased thiophlav S-positive amyloid plaques (Fig 5E) as well as GFAP-positive gliosis around the plaques were
Figure 3. KLK7 is involved in the Aβ degradation activity.

A Inhibition of Aβ degradation activity of CCF-STTG1 cells with MAB2624, a KLK7-neutralizing antibody. Quantification of the relative remaining Aβ40 and Aβ42 in a mixture of normal culture medium and conditioned medium is shown below the blot (n = 3, mean ± s.e.m., *P < 0.05 by Student’s t-test).

B Dose-dependent inhibition of Aβ degradation activity of CCF-STTG1 cells with MAB2624. Quantification of relative remaining Aβ40 in the mixture of normal culture medium and conditioned medium is shown below the blot (n = 3, mean ± s.e.m., **P < 0.01 by Tukey’s test).

C Aβ degradation activity in the conditioned medium of COS-1 cells expressing human KLK7. Quantification of relative remaining Aβ40 in the mixture of normal culture medium and conditioned medium is shown below the blot (n = 3, mean ± s.e.m., ***P < 0.001 by Tukey’s test).

D Aβ degradation activity of the recombinant KLK7 protein. Immunoblot analysis of the remaining Aβ in the mixture of normal cultured medium and conditioned medium of 7PA2 cells, and recombinant human KLK7 and KLK6 protein is shown.

E In vitro degradation of preformed Aβ fibril by purified MBP-tagged hKLK7 protein. Amounts of Aβ fibrils were measured by thioflavin T fluorescence, and relative fluorescence levels at each time point were shown (n = 3, mean ± s.e.m., *P < 0.05, **P < 0.01 by Tukey’s test).

Source data are available online for this figure.
These results indicate that Klk7 is a crucial component of brain Aβ pathology. In this study, we identified that KLK7 is a crucial astrocytic key component in the regulation of brain Aβ economy and attenuates brain amyloid pathology in AD model mice.

**Activation of Klk7 expression by Aβ treatment and memantine**

KLK7 is a terminal protease in the kallikrein-related peptidase cascade and is directly activated by KLK5-mediated prodomain removal (Sotiropoulou et al., 2009). Importantly, the upregulation of KLK5 and KLK7 activity by loss of the endogenous KLK inhibitor serine peptidase inhibitor Kazal type 5 (SPINK5) causes atopic dermatitis-associated diseases, including Netherton syndrome (Furio & Hovnanian, 2014). However, Klk7 mRNA expression is selectively regulated by cytokines in the skin (Morizane et al., 2012), suggesting that transcription of Klk7 mRNA is controlled by a specific mechanism. In fact, Klk7 mRNA expression was significantly and selectively increased in AppΔE9 transgenic mice (Fig 6A). Moreover, mRNA expression of Klk7, but not other Aβ-degrading enzymes (i.e., matrix metalloproteases, neprilysin, and insulin-degrading enzyme), was further specifically augmented in an age-dependent manner (Fig 6B and Appendix Fig S9). Intriguingly, treatment of primary astrocytes with Aβ42, but not with lipopolysaccharide, significantly increased the expression of Klk7 (Fig 6C and D). Although we are unable to exclude the possibility that lipopolysaccharide has some regulatory role in the Klk7, these results suggest that Klk7 transcription was selectively modulated by Aβ in the astrocytes and that selective augmentation of Klk7 expression is possible.

We recently investigated the effect of the memantine, which is the Food and Drug Administration-approved anti-dementia drug and an N-methyl-D-aspartate receptor antagonist, on the Aβ metabolism in the primary cultures (Ito et al., 2017). We noticed that the memantine treatment significantly reduced the spiked human Aβ only in the conditioned medium of primary neuron and glia coculture, but not in that of primary neuronal culture (Fig 7A and B). In addition, we as well as others have found that the chronic treatment of Tg2576 APP transgenic mice with memantine reduced Aβ levels (Dong et al., 2008; Ito et al., 2017). Thus, we hypothesized that memantine would affect Klk7 mRNA expression in the astrocytes and Aβ deposition in the brain. Supporting this notion, Klk7 mRNA level was specifically upregulated in the brains of memantine-treated Tg2576 mice (Fig 7C). Moreover, memantine increased the Klk7 mRNA in the primary astrocytes. However, the N-methyl-D-aspartate receptor antagonist MK-801 failed to induce Klk7 mRNA in the primary astrocytes. In addition, the chronic treatment of Tg2576 APP transgenic mice with memantine reduced Aβ levels (Dong et al., 2008; Ito et al., 2017). This effect was abolished in the astrocytes from Klk7-knockout mice (Fig 7F). These data indicate that astrocytic Klk7 expression is regulated by Aβ in the murine brain and is selectively upregulated by memantine.

**Discussion**

In this study, we identified that Klk7 is a crucial astrocytic key component in the regulation of brain Aβ economy. We observed 1.4-fold to twofold increase of endogenous Aβ levels in the brains of Klk7−/− mice, which is almost comparable to the effects by genetic deletion of neprilysin or insulin-degrading enzyme in vivo (Saido & Leissring, 2012). Moreover, Klk7 deletion increased the thioflavin S-positive amyloid deposition in AD model mice. Thus, KLK7 is one of critical Aβ-degrading enzymes, and this indicates that astrocytes are involved in regulation of Aβ pathogenesis via Klk7 pathway. It was reported that Klk7 is capable to cleave the hydrophobic core motif of Aβ fibrils and reduces the cell toxicity in vitro (Shropshire et al,
Consistent with this result, we found that AppNL-G-F/NL-G-F; Klk7/C0 mice showed the accelerated neuritic changes and inflammatory responses (i.e., increased tau phosphorylation, dystrophic neurites, and reactive astrocytes). However, we did not observe notable change in the number of neurons in the brains and the behavior of congenic mice, probably due to the young age of the model mice. Nevertheless, it remains unclear whether upregulation of KLK7 in the brain causes noxious effect by degrading the other substrates. It is important to understand the physiological function and substrate of KLK7 in the brain by a proteomic approach (Yu et al., 2015).

Aβ treatment selectively induced the expression of Klk7 in vitro, and the Klk7 expression was correlated with Aβ deposition in AD model mice. Molecular mechanism of upregulation of Klk7 mRNA by Aβ remains unclear. However, KLK7 has been implicated in the skin inflammation (Furio & Hovnanian, 2014; Prassas et al., 2015). Intriguingly, anti-inflammatory Th2 cytokines, such as interleukin-4 and interleukin-13, increase KLK7 mRNA expression in human keratinocytes (Morizane et al., 2012). These data suggest that Klk7 expression is homeostatically regulated by Aβ-induced inflammatory response in the astrocytes. Notably, expression of the other Aβ-degrading enzymes, matrix metalloprotease-2, and matrix metalloprotease-9 was also increased in the reactive astrocytes (Yin et al., 2006), although the level of insoluble Aβ was not affected in the brains of Mmp2 or Mmp9 knockout mice. This also suggests that KLK7 plays a primary role in the clearance of aggregated, oligomeric form of Aβ in vivo. Nevertheless, modulation of astrocytic response in the astrocytes to increase KLK7 expression would be a novel therapeutic approach to reduce Aβ deposition (De Strooper & Karran, 2016).

However, in contrast to the model mice, we found that KLK7 mRNA was significantly decreased in AD patients with significant Aβ deposition in the brain. Importantly, the AD model mouse did not reflect all hallmarks of human AD brain (e.g., formation of

![Figure 5](emomolmed.10:e8184/f5.jpg)

**Figure 5. Klk7 gene deficiency increases the brain Aβ levels and amyloid pathology in AppNL-G-F/NL-G-F mice.**

A–C Biochemical analyses of human Aβ in Tris buffer-soluble (A), SDS-soluble (B), and formic acid-soluble (C) fractions of the brains of 3-month-old male AppNL-G-F/NL-G-F; Klk7/C0 mice. Arrowheads indicate total Aβ. Quantification of relative levels of Aβ is shown in Appendix Fig S6.

D Immunohistochemical analysis of the brains of 3-month-old male AppNL-G-F/NL-G-F; Klk7/C0 mice using the 82E1 antibody (red). Magnified images were shown below. Scale bar, 100 μm.

E Quantification results of cortical 82E1-positive total Aβ plaques (left) and the cortical thioflavin S-positive Aβ plaques (right) are shown (n = 3 or 4, mean ± s.e.m., ***P < 0.001 by Student’s t-test).

Source data are available online for this figure.
tangles and major neuronal loss). Thus, these pathological changes might affect the expression of KLK7 mRNA. Moreover, longitudinal studies revealed that Aβ deposition starts 10–15 years before onset of clinical symptoms (De Strooper & Karran, 2016). Thus, other possibility is that the prolonged inflammatory response by Aβ deposition might alter the phenotypes of astrocytes to reduce the KLK7 expression in human brains, although the mechanism underlying this pathological phenotype remains unclear. Notably, expression of KLK7 mRNA was regulated by methylation of histones in cancer cell lines (Raju et al, 2016). It would be important to analyze epigenetic regulation of KLK7 and/or activation status of the astrocytes in the AD brains.

Most important finding of this study is that Klk7 expression was increased by memantine treatment in vitro and in vivo. Memantine would have a great potential in combination therapy to facilitate amyloid clearance, for instance in addition to immunotherapy or even as monotherapy after successful removal of amyloid by immunotherapy. Obtaining mechanistic insight into the selective Klk7 induction by memantine would pave the road toward developing astrocyte-targeted AD therapeutics. Notably, authentic N-methyl-D-aspartate receptor antagonist MK-801 failed to affect the Klk7 mRNA expression, whereas glutamate as well as N-methyl-D-aspartate decreased the Klk7 level. This result suggested the possibility that the N-methyl-D-aspartate receptor in astrocyte shows a different response for these antagonists to that in neurons. In fact, unusual subunit composition of glial N-methyl-D-aspartate receptors distinct from that in neurons has been highlighted (Dzamba et al, 2013), raising the possibility that MK-801-insensitive novel astrocytic N-methyl-D-aspartate receptor is involved in the regulation of Klk7 expression. Intriguingly, memantine induced the expression of Th2 cytokines in T cells irrespective of traditional N-methyl-D-aspartate receptor expression (Kahlfuss et al, 2014). Thus, memantine might modulate anti-inflammatory cytokine signaling in...
astrocytes to increase Klk7 expression via novel mechanism. Importantly, significant adverse effects of memantine on the skin have not been reported, suggesting the possibility that memantine treatment was not sufficient for the skin disease phenotype by overactivation of Klk7 (Hansson et al., 2002). Nevertheless, understanding the regulatory mechanism of Klk7-mediated Aβ degradation at the molecular level should provide novel insights into the pathological role of astrocytes, as well as their possibility as a therapeutic target against AD.

Materials and Methods

Analysis of brains of AD patients

The patient demographics are shown in Appendix Fig S2A. Total RNA from brain tissues was extracted with a TRizol Plus RNA Purification System (Life Technologies) from frozen brain tissues of the frontal cortex. The RNA integrity number (RIN) was determined by a 2100 Bioanalyzer (Agilent Technologies). Samples were subjected to RT-qPCR amplification with a TaqMan Gene Expression Assay (Life Technologies) on an ABI PRISM 7900 HT instrument (Applied Biosystems, Carlsbad, CA, USA) as previously described (Miayshita et al., 2014). Relative gene expression levels were calculated as the cycle difference by means of the delta-delta Ct method using three internal controls genes including GUSB, RPS17, and CASC3 (Appendix Fig S2B) (Miayshita et al., 2014). The subjects were neuropathologically grouped according to the neurofibrillary tangle staging of Braak and Braak (Braak & Braak, 1991).

Data availability

We analyzed two public RNAseq datasets deposited at AMP-AD knowledge portal (https://www.synapse.org/#!Synapse:syn2580853): the Mayo sample set (Allen et al., 2016) and MSBB studies. The Mayo study comprises 156 temporal cortex samples from 80 patients with AD and 76 controls. We assessed Klk7 expression of the temporal cortex between AD patients and controls by a simple model adjusting for key covariates: age at death, gender, RIN, source, and flow cell. For the MSBB study, we selected 228 samples from BM22 and 206 samples from BM36 excluding the samples without the information of Braak NFT stage and RIN. We downloaded raw read-count data and applied them to the R package, DESeq2 (version 1.14.1) to explore alteration of gene expression associated with the amyloid plaque burden. More specifically, we divided degrees of neuritic plaque density into five categories at equal intervals and compared gene expression levels among the five categories adjusting for age at death, gender, race, and RIN.

Animals

AppNL-GF/NL-GF mice (Saito et al., 2014) were originally generated at RIKEN Brain Science Institute, Japan. The sperm of Klk7<sup><small>−/−</small></sup> (KOMP)Veg/+ heterozygous mouse (project ID: VGl4816) used for this study was generated by the trans-NIH Knockout Mouse Project (VelociGene at Regeneron Inc. (U01HG004085) and the CSD Consortium (U01HG004080)) and obtained from the KOMP Repository at UC Davis and CHORI (U42RR024244) (www.komp.org). In vitro fertilization using the sperm was achieved at the Center for Disease Biology and Integrated Medicine, Graduate School of Medicine, The University of Tokyo. Maintenance of Tg2576 mice (Hsiao et al., 1996) and chronic administration of memantine (20 mg/kg/day) to 8-month-old female Tg2576 mice for 1 month by p.o. were performed at Daiichi-Sankyo Co. Ltd. Genomic DNAs of mice were obtained from tails by alkaline lysis. Genotypes were determined by genomic PCR using following primers: 5′-gacgcacctgttcacacacattc-3′ (Reg-Neof) and 5′-accacacacacagctctccttc-3′ (Reg-Klk7-R) for Klk7 knockout locus; 5′-ctgaggctactcgcctgg-3′ (Reg-Klk7-wtR) and 5′-cagagtcgcgagaagtaaggg-3′ (Reg-Klk7-wtF) for Klk7 wild-type locus; 5′-atctcggagaagtagtg-3′ (E16WT) and 5′-tgatagtgagacatcagac-3′ (WT) for App wild-type locus; and 5′-atctcggagaagtagtg-3′ (E16MT) and 5′-ctgatatagctatagaaag-3′ (loxP) for App knockin locus.

Antibodies and chemicals

The following primary antibodies were used in this study: anti-human Aβ 82E1 (1:2,500, IBL #10323), anti-human Aβ 6E10 (1:2,000, BioLegend #SIG-39300), anti-KLK7 (1:2,000, Abcam ab28309), anti-KLK7 antibody for neutralization (20 µg/ml, R&D Systems MAB2624), anti-KLK7 for immunostaining (1:40, R&D Systems AF2624), anti-neprilysin/CD10 (1:2,000, R&D Systems AF1126), anti-IDH (1:2,000, Merck Millipore ST1120), anti-GFAP (1:1,000, Sigma-Aldrich G3893), anti-Alldh1L1 for immunostaining (1:5, NeuroMab 75-164), anti-Aldh1L1 for immunoblotting (1:5, NeuroMab 75-140), anti-BACE1 BACE1c (1:1,000, IBL #18711), anti-APP ApPC (1:1,000, IBL 18961), anti-nicastrin N1660 (1:1,000, Sigma-Aldrich N1660), anti-ADAM10 (a kind gift from Dr. Paul Saftig) (Suzuki et al., 2012), anti-calnexin C-terminus (1:1,000, Enzo Life Sciences ADI-SPA-860), anti-total Tau Tau-5 antibody (1:1,000, BioLegend # SIG-39413), anti-Phospho-PHF-tau pThr231 AT180 antibody (1:1,000, Invitrogen MN1040), Anti-Phospho-PHF-tau pThr181 AT270 antibody (1:1,000, Invitrogen MN1050), anti-MMP-9 TP221 (1:1,000, Torrey Pines Biolabs), and anti-α-tubulin (1:5,000, Sigma-Aldrich DM1A). For isotype control of MAB2624 (mouse IgG2a), we used same concentration (final 30 µg/ml) anti-V5 Tag (Thermo Fisher #R960-25) and anti-LR11 (BD Biosciences #611860) monoclonal antibodies.

The following chemicals were used in this study: thiolaflavin S (Sigma-Aldrich, stock solution was dissolved in 50% ethanol, final concentration was 0.05%), diisopropyl fluorophosphates (WAKO, stock solution was dissolved in 2-propanol, final concentration was 0.5 mM), E-64 (Roche Applied Science, stock solution was dissolved in 50% ethanol, final concentration was 10 µg/ml), ethylenediaminetetraacetic acid (DOJINDO, stock solution was dissolved in distilled water, final concentration was 10 mM), phosphoramidon (Nacalai Tesque, stock solution was dissolved in distilled water, final concentration was 10 µg/ml), pepstatin A (Sigma-Aldrich, stock solution was dissolved in 10% acetic acid, final concentration was 1 µg/ml), complete protease inhibitor cocktail (Roche Applied Science), PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science), s2-anti-plasmin (WAKO, stock solution was dissolved in distilled water, final concentration was 70 µg/ml), acetylthiemione (Tokyo Chemical Industry, stock solution was dissolved in distilled water, final concentration was 1 mM), tosyl-L-lysyl-chloromethane hydrochloride (WAKO, stock solution was dissolved in ethanol, final concentration was...
100 µg/ml), tosyl phenylalanyl chloromethyl ketone (WAKO, stock solution was dissolved in DMSO, final concentration was 100 µg/ml), GM6001 (Enzo Life Science, stock solution was dissolved in DMSO, final concentration was 25 µM), MK-801 (Sigma-Aldrich, stock solution was dissolved in distilled water, final concentration was 10 µM), N-methyl-D-aspartate (Sigma-Aldrich, stock solution was dissolved in distilled water, final concentration was 50 µM), L-glutamine (KANTO, stock solution was dissolved in distilled water, final concentration was 20 µM), lipopolysaccharide from *Escherichia coli* (Imgenex, stock solution was dissolved in distilled water, final concentration was 1 µg/ml), and DRAQ5™ far-red fluorescent DNA dye (ImmunoChemistry Technologies, final concentration was 1:1,000 dilution). Memantine (stock solution was dissolved in distilled water, final concentration was 30 µM) was provided by Daiichi-Sankyo Co. Ltd.

**Cell culture and transfection**

CCF-STTGG1, U-87, and BE(2)-C cells were purchased from European Collection of Authenticated Cell Cultures. MG-6 cell (Take-nouchi et al., 2005; Nakamichi et al., 2006) was provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. COS-1 (Maruyama et al., 1990), HEK293 (Morigashii et al., 2002), Neuro2a (Tomita et al., 1997), H4 (Asai et al., 2010), and SH-SY5Y (Takasugi et al., 2003) cells were kindly provided from Dr. Kei Maruyama (Saitama Medical University). BV-2 (Blasi et al., 1990) and 7PA2 cells (Podlisny et al., 1990) were gifts from Drs. Makoto Michikawa (Nagoya City University) and Edward Koo (University of California, San Diego), respectively. Cells were cultured in Dulbecco’s modified Eagle’s medium with high glucose (WAKO) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone), 50 units/ml penicillin (Invitrogen), and 50 mg/ml streptomycin (Invitrogen) at 37°C under humidified air containing 5% CO2, alamarBlue Cell viability assay (Thermo Fisher Scientific) was performed as manufacturer’s instruction. We routinely check mycoplasma contamination by DAPI staining and PCR analysis. Primary dissociated cortical glial cultures were prepared from mice at postnatal day (P) 1–3 or P18 Wistar rats and maintained in Dulbecco’s modified Eagle’s medium containing 10% FBS, as previously described (Fukumoto et al., 1999; Suzuki et al., 2012). To separate primary astrocyte and microglia, the glial culture was gently shaken for 24 h at 37°C under humidified air containing 5% CO2. Primary microglia detached to the medium were then replated to different plate. Human KLK7 and KLK6 cDNAs were amplified from DNAFORM clone 100008376 (GenBank accession no. DQ939916) and Human brain Total RNA (TaKaRa, #636539), respectively, using KOD-Plus-Neo DNA polymerase (TOYOBO). For the overexpression experiment in mammalian cell, cDNA was inserted into the pEFe6/V5-His-TOPO vector (Invitrogen). Transfection into COS-1 cells using FuGene 6 (Roche Applied Science) was described previously (Suzuki et al., 2012). For purification of MBP-tagged proteins, cDNA was inserted into pMAL-p2x vector (New England Biolabs) (Miyamoto et al., 2011). Plasmids are transformed into *E. coli* Rosetta 2(DE3) (Novagen), and periplasmic expression was induced by 0.1 mM IPTG. Overexpressed proteins were purified by amylase column with maltose according to manufacturer’s instructions.

**Aβ degradation assay**

7PA2 cells were cultured at confluency for 24 h, and their conditioned medium was collected. After centrifugation at 5,900 × g for 10 min at 4°C, the supernatant was stored at −80°C until use. Aβ levels were determined by ELISA or immunoblotting, using the urea/SDS–PAGE gel system, as described previously (Tomita et al., 1997; Ohki et al., 2011). Synthetic Aβ peptides were obtained from Peptide Institute (Aβ40 and Aβ42) and AnaSpec (Aβ37, Aβ38, and Aβ39). For the Aβ degradation assay, the 7PA2 medium was coincubated with the same amount of conditioned medium from CCF-STTGG1, U-87, and primary glial cells at 37°C for 24 h. Recombinant human KLK6 and KLK7 proteins purchased from R&D Systems were diluted with Hank’s balanced salt solution (HBSS). 7 μl of 5 nM synthetic Aβ40 peptide was mixed with 49 μl of HBSS containing KLK proteins at the indicated concentrations and incubated at 37°C for 24 h.

For human Aβ spike experiment, a rat primary neuron culture was obtained by the treatment of 2 μM of AraC to the primary cells isolated from rat brain on days in vitro 1 as previously described (Fukumoto et al., 1999; Suzuki et al., 2012). The primary culture without AraC treatment was defined as the neuron + glia culture. At days in vitro 8, 30 μM of memantine or water was added to the cultures. At days in vitro 14, 30 nM of synthetic human Aβ42 peptide was added to the primary neuron or neuron/glial culture. At days in vitro 15, levels of remaining human Aβ42 in the conditioned medium were measured using a human Aβ-specific sandwich ELISA kit (#296-64401, WAKO) (n = 6, mean ± s.e.m.).

**Injection of recombinant proteins into the hippocampal region of mouse brains**

MBP and MBP-hKLK7 proteins were injected into the hippocampi (Hori et al., 2015) (anterior–posterior −2.5 mm, medial–lateral ±2.0 mm, dorsal–ventral −1.8 mm from Bregma) in 5-month-old wild-type mice. Three hours after injection, the hippocampi were extracted and homogenized in Tris buffer. Aβ40 level in Tris-soluble fraction was measured by two-site ELISA system, Human/Rat β-Amyloid (40) ELISA Kit (#294-62501, WAKO).

**Immunocytochemistry and immunohistochemistry**

Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (8 mM Na2HPO4, 2 mM NaH2PO4, and 131 mM NaCl) for 20 min, incubated in phosphate-buffered saline containing 0.1% Triton X-100 for 15 min, and stained for 2 h with primary antibodies at room temperature. The cells were then washed and incubated for 1 h with Alexa Fluor-conjugated secondary antibodies (Invitrogen), 4′,6-diamidino-2-phenylindole (DAPI), and/or DRAQ5. For immunohistochemistry, 3-month-old mice were anesthetized and perfused with 10 ml PBS, sacrificed by decapitation, and their brains fixed by soaking them in PBS containing 4% paraformaldehyde overnight. The brains were then rinsed in PBS and then serially dehydrated in 70, 80, 90, and 99% ethanol (WAKO), and then, the ethanol was replaced by incubation in xylene (WAKO) twice and then further replaced by incubation in paraffin (WAKO) three times. Paraffin sections (4 µm) were prepared using a microtome (MicroEdge Instruments, Inc.). Paraffin sections were soaked in xylene three times for 5 min, in ethanol (99, 80, and 70%) for 1 min.
Preparation of samples for biochemical analyses

For total cell lysates, cells were lysed in 2% SDS, briefly sonicated, and then incubated at 37°C for 20 min with 2% mercaptoethanol.

Mouse brains were homogenized in 10× volumes of Tris buffer (50 mM Tris–HCl pH 7.6, 150 mM NaCl, complete protease inhibitor cocktail, and PhosSTOP phosphatase inhibitor cocktail) with 25 strokes using a mechanical homogenizer and centrifuged at 200,000 × g for 20 min at 4°C. The resultant supernatant was collected as the brain Tris buffer-soluble fraction. After addition of the same amount of 2% Triton X-100/Tris buffer, the pellet was centrifuged at 200,000 × g for 20 min at 4°C. The resultant supernatant was collected as the brain Triton X-fraction. Then, the same amount of 2% SDS containing Tris buffer was added to the pellet. After homogenization at room temperature, the pellet was incubated for 2 h at 37°C and centrifuged at 200,000 × g for 20 min at 20°C. The resultant supernatant was collected as the brain SDS fraction. Finally, the pellet was sonicated (BRANSON; output: 2, duty cycle: 90, 15 s) with 500 μl of 70% formic acid (WAKO) solution. Samples were centrifuged at 200,000 × g for 20 min at 4°C, and the resultant supernatant was freeze-dried for 2 h (Thermo Scientific, Savant RVT5105). The pellet was dissolved in the same volume of DMSO (WAKO) as the brain weight and stored at −80°C until use. Protein concentrations of the fractions were measured by the BCA protein assay (Pierce).

Immunological analysis and quantitation of immunoblot band densities

Aβ levels were measured by ELISA using the Human/Rat β-Amyloid (40) ELISA Kit (#294-62501, WAKO) and the Human/Rat β-Amyloid (42) ELISA Kit, High Sensitivity (#292-64501, WAKO) or immunoblotting. For immunoblotting, samples were dissolved in Laemmli sample buffer [final concentration of 1 M Tris–HCl pH 6.8, 20% SDS, 30% glycerol, 1% Brilliant Green (WAKO), 1% CBB-G250 (Nacalai Tesque)] and separated by SDS–PAGE. Gels were transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore). The membranes were incubated in 5% skim milk or PVDF Blocking Reagent for Can Get Signal® (TOYOBO), treated with primary antibodies, and then probed with horseradish peroxidase (HRP)-conjugated secondary antibodies (GE Healthcare and Jackson ImmunoResearch), and chemiluminescent signals were acquired using ImageQuant LAS 4000 (GE Healthcare). Band intensities were measured using ImageJ software (NIH). The ratio of each of the proteins to α-tubulin or calnexin was acquired and normalized to the value of the control.

Quantitative real-time PCR analysis for cultured cell and model mouse

Total mRNAs were isolated and purified from cell cultures using ISOGEN reagent (Nippon Gene). 1 μg of purified mRNAs from each sample was reverse-transcribed into cDNA using ReverTra Ace qPCR RT kit (TOYOBO). cDNA templates (1 μl) were used for the real-time PCR reaction and relative quantification using LightCycler 450 (Roche) using the SYBR Green I protocol. The amount of target mRNA was normalized to Gapdh mRNA in each sample. The following primer pairs were used to detect the indicated target genes: 5′-atgggcaatgtcactcctg-3′ (forward) and 5′-gctgcggctcagaggtt-3′ (reverse) for Klk5; 5′-tgctggctgtttgtcacta-3′ (forward) and 5′-tgtgcttgctgtcatctgca-3′ (reverse) for Klk6; 5′-tgctgcggctcagcttacct-3′ (forward) and 5′-gtctggcttgcttgtggca-3′ (reverse) for Klk7; 5′-gtggcttgctgtcacttacct-3′ (forward) and 5′-tgtgcggcttgctgtcagct-3′ (reverse) for Mme; 5′-ttgctgcggctgcttacct-3′ (forward) and 5′-ctggagctgcgctgcctgct-3′ (reverse) for Spin5; 5′-gtggcttgctgtcacttacct-3′ (forward) and 5′-gtggcttgctgtcacttacct-3′ (reverse) for Ile; 5′-ctgagcagcagacaataag-3′ (forward) and 5′-ctgagcagcagacaataag-3′ (reverse) for Mmp-9; and 5′-tgtggcttgctgtcacttacct-3′ (forward) and 5′-tgtggcttgctgtcacttacct-3′ (reverse) for Gapdh.

Statistical analysis

For analyses of human samples, statistical analyses were performed by Mann–Whitney U-test as previously described (Miyashita et al., 2014). All samples were analyzed in blind and randomized manner. For quantitative immunoblot analysis, immunofluorescence, and qRT–PCR in cells and mice, Student’s t-test was used for comparisons between two-group data, and Tukey’s test was used for multiple group comparisons. Statistical analyses were performed by KPyPlot or Excel software. In figures, statistical significance is indicated by *P < 0.05, **P < 0.01, and ***P < 0.001. All P-values are shown in Appendix Table S1.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the Institutional Review Board of Niigata University. All animal experimental procedures were performed in accordance with the guidelines for animal experiments of The University of Tokyo or Daichi-Sankyo Co., Ltd., and were approved by the Institutional Animal Care and Use Committee/ethics committee of the Graduate School of Pharmaceutical Sciences, The University of Tokyo (protocol no. P25-6) or Daichi-Sankyo Co., Ltd., Tokyo, Japan.

Informed consent

Informed consent was obtained from all subjects. The written informed consent was received from participants prior to inclusion in the study. Participants were identified by number, not by name.
The paper explained

**Problem**
Several lines of evidence suggest that the decreased clearance of Aβ from brain is related to the pathogenesis of Alzheimer’s disease (AD). However, the precise role of astrocytes, which is most abundant cell type in the brain, in Aβ clearance remains unclear.

**Results**
Here, we identified kallikrein-related peptidase 7 (KLK7) as an astrocyte-derived Aβ-degrading enzyme that cleaves not only monomer but aggregated forms of Aβ. KLK7 mRNA level was significantly reduced in AD brain, and genetic ablation of KLK7 in AD model mice exacerbated the amyloid pathology. We also found that anti-dementia drug memantine can increase its expression and Aβ degradation activity of astrocytes. Thus, memantine would have a great potential to facilitate the astrocyte-mediated proteolytic clearance of the brain amyloid.

**Impact**
Our study would provide novel insights into the pathological role of astrocytes in AD, as well as their possibility as a cellular target in the development of anti-Aβ therapeutics.

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