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Developmental retardation, microcephaly, and peptiduria in mice without aminopeptidase P1

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ABSTRACT

Cytosolic aminopeptidase P1 (APP1) is one of the three known mammalian aminopeptidase Ps (APPs) that cleave the N-terminal amino acid residue of peptides in which the penultimate amino acid is proline. In mammals, many biologically active peptides have a highly conserved N-terminal penultimate proline. However, little is known about the physiological role of APP1. In addition, there is no direct evidence to associate a deficiency in APP1 with metabolic diseases. Although two human subjects with reduced APP activity exhibited peptiduria, it is unclear which of the three APP isoforms is responsible for this disorder. In this study, we generated APP1-deficient mice by knocking out Xpnpep1. Mouse APP1 deficiency causes severe growth retardation, microcephaly, and modest lethality. In addition, imino-oligopeptide excretion was observed in urine samples from APP1-deficient mice. These results suggest an essential role for APP1-mediated peptide metabolism in body and brain development, and indicate a strong causal link between APP1 deficiency and peptiduria.

1. Introduction

Many biologically active peptides in mammals have a highly conserved proline residue at the penultimate N-terminal position. This configuration forms an X-Pro motif in which X is any amino acid. More than 23 bioactive peptides with this structure, including cytokines, growth hormones, and neuropeptides, have been identified [1]. The unique cyclic structure of proline in the X-Pro motif confers both resistance to non-specific proteolytic degradation and biological activity [2].

Proteolytic cleavage of the first residue in the X-Pro motif is mediated by aminopeptidase Ps (APPs), which are also known as X-prolyl aminopeptidases (EC 3.4.11.9). APPs have been found in a wide range of organisms including vertebrates, insects, nematodes, bacteria, and plants [3–7]. There are three known APPs (APP1-3) in mammals. Each APP isoform is encoded by a distinct gene (Xpnpep1-3) and has a specific subcellular localization. Soluble cytosolic isoform APP1 has been identified in human leukocytes [8], human platelets [9], rat brain [10], and guinea pig serum [11]. This enzyme exhibits broad substrate specificity for peptides of diverse sizes [10]. In contrast, APP2 is a glycosylphosphatidylinositol (GPI)-anchored ectoenzyme that is ubiquitously expressed in human tissues [12,13]. In comparison to APP1, APP2 has a much more restricted substrate specificity [14,15]. The third isoform, APP3, is mainly localized to mitochondria and shows a ubiquitous tissue distribution pattern in humans [16,17].

Deficiency in APP activity has been known to cause human peptiduria, which is the massive urinary excretion of undigested imino-oligopeptide. In addition, one human subject that was deficient in APP activity exhibited severe developmental retardation and microcephaly [18], suggesting that APP activity might be critical for the regulation of body and brain development. However, a direct causal relationship between APP deficiency and developmental retardation has not been established in vivo. Additionally, it is still not known which of the three APP isoforms is responsible for such dysfunction in these subjects.

In this study, we genetically disrupted mouse APP1 function and investigated the consequences. APP1-deficient mice displayed severe developmental retardation and microcephaly. In addition, APP1 deficiency caused peptiduria in mice. Our results indicate a strong causal link between APP1 deficiency and developmental dysfunction as well as peptiduria, and we identify APP1 as an important regulator of body and brain development.

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2. Materials and methods

2.1. Generation of Xpnpep1 knockout mice

A mouse embryonic stem (ES) cell line (strain 129 Sv/Ev) containing a gene-trap in the Xpnpep1 gene was provided by Dr. Philippe Soriano (Fred Hutchinson Cancer Research Center, Seattle, WA). The ES cells were injected into blastocysts from C57BL/6J mice to generate chimeric individuals, which were then crossbred with C57BL/6J females to obtain heterozygous Xpnpep1 mutants (N1). Germline transmission and mouse genotypes were monitored by PCR of the genomic DNA using the oligonucleotide primers 5′-CCTTAGACGTGTCCCGAGGT-3′ and 5′-TGCAGCCTAGAGGAAGGACA-3′ (wild-type) or 5′-CCTGGACTACTGCGCCCTAC-3′ (β-geo). The heterozygous mice were backcrossed to C57BL/6J and 129S4/SvJae for 5–7 generations. All analyses were performed on littermates of both genotypes that were derived from intercrosses between C57BL/6J and 129S4/SvJae heterozygous parents. Maintenance of all animals and related experiments were performed according to institutional guidelines for the care and use of animals in research (SNU-101217-2).

2.2. Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was prepared from 4-week-old mice brains using the RNeasy Mini Kit (Qiagen, CA, USA). First strand cDNAs were synthesized from the total RNA with random hexamer primers using AMV reverse transcriptase (NEB, MA, USA). cDNA was PCR-amplified using primers specific to Xpnpep1 (5′-GGTGACACCCTCAGGAGACAAG-3′ and 5′- TGGATCCTTCTGGAAGCACACT-3′), Xpnpep1-β-geo (5′-GGTGACACCCTCAGGAGACAAG-3′ and 5′-GCCATGTCACAGATCATCAAGC-3′) and GAPDH (5′-ACAGCAACTCCCACTCTTCCAC-3′ and 5′-AGTTGGGATAGGGCCTCTCTTG-3′).

2.3. X-gal staining

Four-week-old mice were anesthetized and transcardially perfused with phosphate-buffered saline (PBS), followed by treatment with a fixative containing 4% (w/v) paraformaldehyde in PBS. Mouse brain sections (in 200 μm slices) were incubated in X-gal staining solution (5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% deoxycholate, 0.02% NP-40 and 1 mg/ml X-gal in PBS) at 37 °C overnight. Images were acquired using a microscope (BX51WI, Olympus, Japan) equipped with a cooled charge-coupled device camera (DP70, Olympus, Japan).

2.4. Antibodies and immunoblotting

Rabbit polyclonal anti-APP1 antibodies were raised against a synthetic peptide that mimicked the amino acid sequence of mouse APP1 (CAKTKYNFNNRGSLT). For immunoblotting, protein extracts were prepared from 6-week-old male littermates. Homogenates were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and blotted to nitrocellulose membranes. Signals were detected with enhanced chemiluminescence (GE Healthcare, UK).

2.5. MALDI-TOF MS analysis

Mouse urine samples were analyzed with a 4800 MALDI-TOF/TOF analyzer in the positive-ion mode (Applied Biosystems, CA, USA) using the dried droplet method. Samples were dissolved in HPLC-grade water. When CHCA (alpha-cyano-4-hydroxycinnamic acid, Bruker Daltonik GmbH, Germany) was used as a matrix, 1 μl of sample was diluted with an equal volume of CHCA (10 mg/ml in acetonitrile–trifluoroacetic acid-water; 70:0.5:29.5, v/v/v), and 0.25 μl of this mixture was loaded onto a MALDI target plate. Spectra were accumulated over a range of 10–500 *m*/*z*. Five hundred scans were accumulated for each mass spectrum. Data were acquired in the reflectron mode and processed using Data Explorer (Applied Biosystems, CA, USA).

2.6. Statistical analysis

Statistical analyses were performed using IGOR Pro (WaveMetrics, OR, USA) and SPSS (Statistical Package for the Social Sciences, IBM, NY, USA) software. All data were compared using a parametric two-tailed Student's *t*-test and nonparametric Mann–Whitney-Wilcoxon test. All data and bars in the figures are expressed as the mean ± s.e.m.

3. Results

3.1. Generation of Xpnpep1 knockout mice

To investigate the physiological roles of APP1 in vivo, we generated Xpnpep1 knockout (KO) mice using a gene-trapped ES cell line that harbors a gene-trap cassette within intron 3 of Xpnpep1 (Fig. 1A). Offspring genotypes were identified by PCR, which yielded products of approximately 300 and approximately 600 base pairs for the wild-type (WT) and gene-trapped alleles, respectively (Fig. 1B). Because APP1 is expressed in the rodent brain [10], we analyzed the efficiency of gene trapping using RT-PCR in the mouse brain. Insertion of the gene-trap cassette into the Xpnpep1 gene produced Xpnpep1-β-geo fusion transcripts, and native Xpnpep1 transcripts were not detected in the Xpnpep1 homozygous mutant ($Xpnpep1^{\beta\text{-geo}/\beta\text{-geo}}$) mice (Fig. 1C). These results demonstrate an absence of functional APP1 transcripts in homozygous mutants (Xpnpep1 KO).

We next examined the suppression of APP1 expression in Xpnpep KO mice at the protein level. Immunoblot analysis with antibodies raised against mouse APP1 detected APP1 signals in wild-type (WT) tissues but not in KO tissues (Fig. 1D). APP1 was expressed in all examined tissues, with the strongest expression found in the intestine, testis, kidney, and brain. Lung and skeletal muscle showed weak APP1 signals. The ubiquitous expression of APP1 in mice is consistent with previous PCR analyses of human cDNA [16]. However, relative expression levels of APP1 protein in mouse tissues show different patterns compared to those of human APP1 transcripts.

3.2. Developmental retardation in Xpnpep1 KO mice

Xpnpep1 KO offspring derived from heterozygous parents of C57BL/6J background showed embryonic or perinatal lethality. No homozygous pups were able to survive beyond 3 days after birth (data not shown). Xpnpep1 KO mice in a hybrid genetic background derived from intercrosses between C57BL/6J and 129S4/SvJae heterozygous parents were viable, but the percentage of Xpnpep1 KO offspring did not conform to Mendelian segregation ratios (WT, 29.5%; HT, 51.7%; KO, 18.8%; $n = 505$ mice). The body size of Xpnpep1 KO pups was smaller than that of WT and heterozygous littermates at birth, and Xpnpep1 KO mice grew more slowly (Fig. 2A). Hybrid background Xpnpep1 KO individuals exhibited modest postnatal lethality during the first 4-week after birth. In addition, we could observe a gender difference in the survival rate of Xpnpep1 KO mice. The survival rate was higher in the female Xpnpep1 KO (45/50 mice) in comparison to the male (31/46 mice) KO mice (Fig. 2B). Considering that the Xpnpep2 gene is located on chromosome X in both humans and mice [19], higher

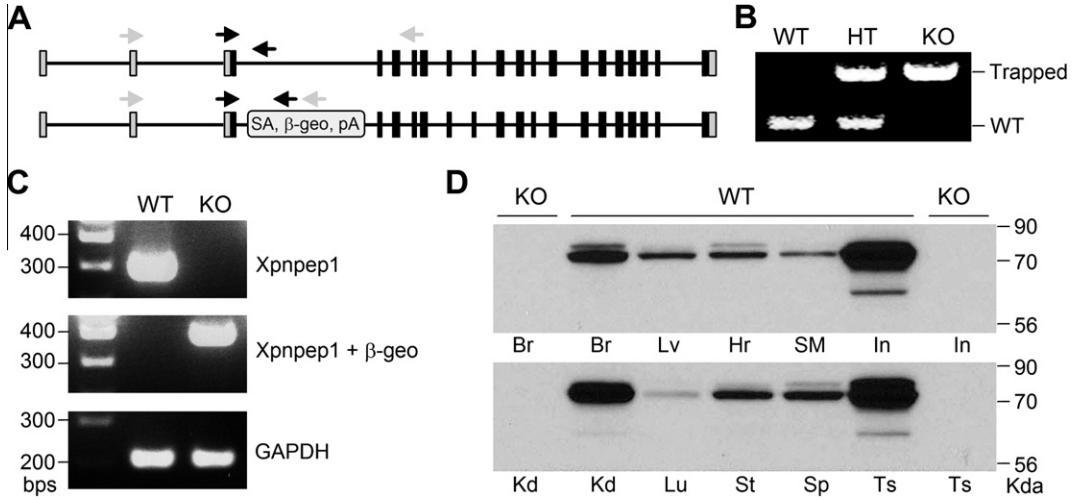

Fig. 1. Generation of Xpnpep1 KO mice. (A) Schematic diagram of wild-type (top) and gene-trapped (bottom) alleles. Gray and black vertical bars indicate non-coding and coding exons, respectively. Black arrows indicate primers used for PCR genotyping and gray arrows indicate RT-PCR primers. SA, splice acceptor; β-geo, β-galactosidase + neomycin phosphotransferase; pA, polyadenylation signal. (B) PCR genotyping of WT, Xpnpep1 HT (heterotype) and Xpnpep1 KO mice. (C) RT-PCR of genotyped WT and Xpnpep1 KO mice. GAPDH was used as a control. (D) Tissue expression patterns of APP1 protein. Equal amounts of total protein were loaded in each lane (10 μg/lane). APP1 proteins are not detected in tissues of Xpnpep1 KO mice. Br, brain; Lv, liver; Hr, heart; SM, skeletal muscle; In, small intestine; Kd, kidney; Lu, lung; St, stomach; Sp, spleen; Ts, testis.

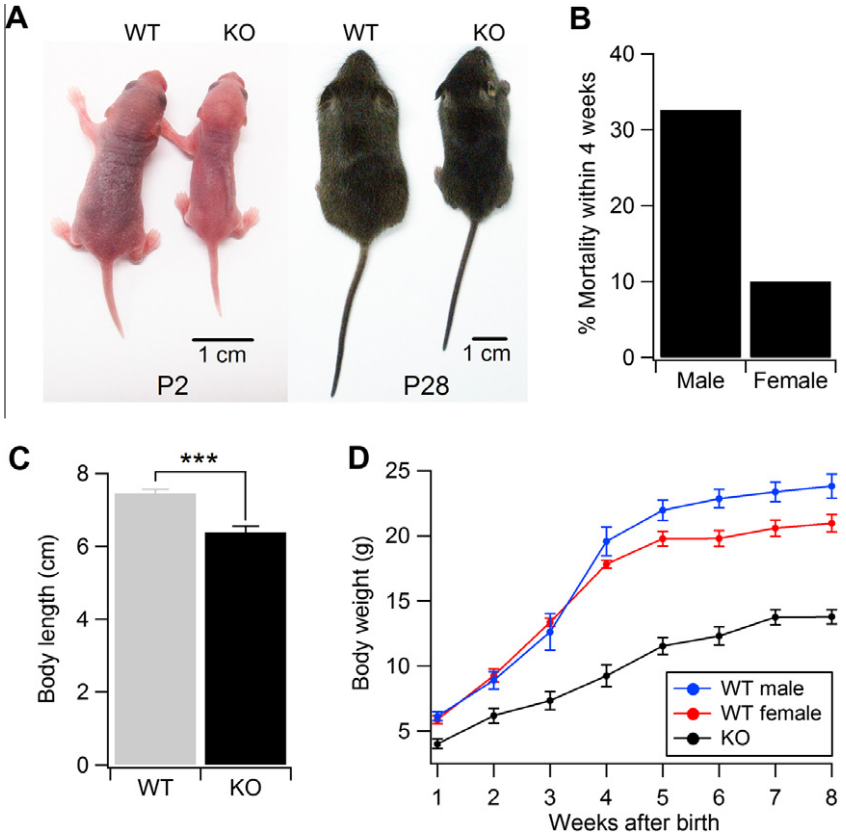

Fig. 2. Xpnpep1 KO mice exhibit severe developmental retardation and lethality. (A) Representative images of male WT littermates and Xpnpep1 KO mice at P2 (left) and P28 (right). (B) Mortality of male and female Xpnpep1 KO mice within 4 weeks. (C) Reduced body size of Xpnpep1 KO mice. (D) Weight curves of WT and Xpnpep1 KO mice show severe developmental retardation in Xpnpep1 KO mice.

survival rates in female KO mice might be attributable to the partial compensation of APP1 function by X-linked APP2.

Surviving Xpnpep1 KO animals were distinguishable from their WT littermates by their smaller size. As shown in Fig. 2C, Xpnpep1 KO animals were approximately 15% shorter in body length than littermate controls (6.38 ± 0.17 vs. 7.44 ± 0.11 cm) at 4-week after birth ($p = 0.00015$, Student's t-test; $p < 0.001$, Mann–Whitney-Wilcoxon test; $n = 10$).

We next examined the weight of WT and Xpnpep1 KO mice to investigate the role of APP1 in body growth. The weights of WT

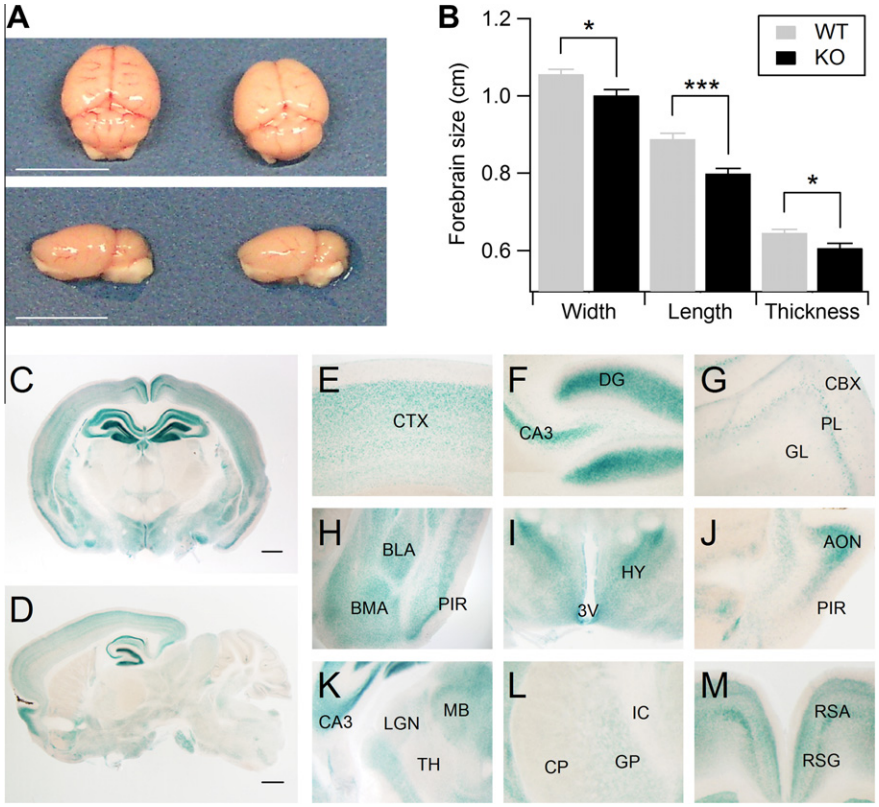

Fig. 3. Microcephaly in Xpnpep1 KO mice. (A) Representative images of male WT littermates and Xpnpep1 KO brains. Scale bar, 1 cm. (B) Quantification of the reduced brain size in Xpnpep1 KO mice. Forebrain width $^{*}p = 0.014$, $p = 0.015$; length $^{***}p = 0.00045$, $p = 0.001$; thickness $^{*}p = 0.025$, $p = 0.023$. $n = 10$. P values were generated by the Student's *t*-test and Mann–Whitney–Wilcoxon test. (C–M) Expression patterns of APP1-β-geo fusion proteins in the mouse brain. Coronal (C) and sagittal (D) brain sections from 4-week-old Xpnpep1 HT mice were stained with X-gal. Scale bar, 1 mm. High-magnification images of sections reveal the expression of fusion proteins in the cortex (E), hippocampus (F), cerebellum (G), amygdala (H), hypothalamus (I), olfactory nucleus (J), midbrain (K), striatum (L) and retrosplenial cortex (M). CTX, neocortex; DG, Dentate gyrus; CBX, cerebellar cortex; PL, Purkinje layer; GL, granular layer; BLA, basolateral amygdalar nucleus; BMA, basomedial amygdalar nucleus; PIR, piriform cortex; 3 V, third ventricle; HY, hypothalamus; AON, anterior olfactory nucleus; LGN, lateral geniculate nucleus; MB, midbrain; CP, caudate putamen; GP, globus pallidus; IC, internal capsule; RSG, retrosplenial granular cortex; RGA, retrosplenial agranular cortex.

male ($n = 5$) and female ($n = 6$) mice were indistinguishable during the first 4-week ($p > 0.05$, Student's *t*-test and Mann–Whitney-Wilcoxon test), but male mice gained more weight than female mice after five-weeks. In contrast, Xpnpep1 KO animals showed no gender difference in body weight during the 8-weeks of study ($p > 0.05$, $n = 3$ male and 6 female mice). In comparison with their WT littermates, which experienced a steep weight gain during the first five-weeks, Xpnpep1 KO mice exhibited slow weight gain (Fig. 2D). At 8-weeks after birth, KO mice body weights were approximately 62.2% of that of WT mice ($p < 0.001$, Student's *t*-test and Mann–Whitney–Wilcoxon test). Together, these results suggest that APP1 is essential for normal development and that a deficiency of APP1 in males is riskier than in females.

3.3. Deficiency of APP1 causes microcephaly in mice

Because the Xpnpep1 KO exhibited a smaller head size than their WT littermates (Fig. 2A), we investigated the role of APP1 in brain development by measuring the brain size of 4-week-old mice. The gross morphology of Xpnpep1 KO brains was normal. However, Xpnpep1 KO mice exhibited significantly reduced brain size next to WT mice (Fig. 3A). Quantitative analysis revealed approximately 10% reduction in the width, length, and thickness of the Xpnpep1 KO forebrain (Fig. 3B).

The APP1 brain expression pattern is unclear in mammals. In cultured brain cells, APP1 activity was mainly detected in astrocytes, rather than in oligodendrocytes or neurons [20]. However, an in situ hybridization study showed intense Xpnpep1 transcript signals in the neurons of various brain regions [21].

Because gene trapping produces APP1 (39 aa)-β-geo fusion proteins in the mutant mice, we investigated the expression patterns of APP1 by monitoring regional and cellular β-galactosidase activities in the brain. The fusion protein was expressed in various brain neurons, with the strongest signals occurring in granule neurons of the hippocampal dentate gyrus (Fig. 3C–M). These results suggest that APP1-mediated peptide metabolism in neurons is important for normal brain development.

3.4. Peptiduria caused by APP1 deficiency

Human APP deficiency has been known to cause peptiduria [18]. However, the APP isoform responsible for peptiduria is unknown. We therefore examined whether APP1 deficiency causes peptiduria in mice. The color of urine samples collected from Xpnpep1 KO mice was slightly darker compared to that of WT mice (data not shown). We analyzed urine samples from WT and Xpnpep1 KO mice with matrix associated laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; Fig. 4A). Because Gly-Pro-4Hyp-Gly, Gly-3Hyp-4Hyp, Gly-Pro-4Hyp, Pro-4Hyp and Gly-Pro peptides were found in human subjects [18], we analyzed the quantities of these imino-oligopeptides from the MALDI-TOF mass spectrum. We calculated that the masses of these aforementioned imino-oligopeptides using PeakView software (AB Sciex) were 342.160, 301.1379, 285.137, 228.123 and 172.085 Da, respectively. The tetrapeptide Gly-Pro-4Hyp-Gly was detected in urine samples of Xpnpep1 KO mice but not in urine from WT samples (Fig. 4B), which was consistent with findings from APP-deficient human subjects. Interestingly, tripeptide Gly-3Hyp-4Hyp was not detected in the ur-

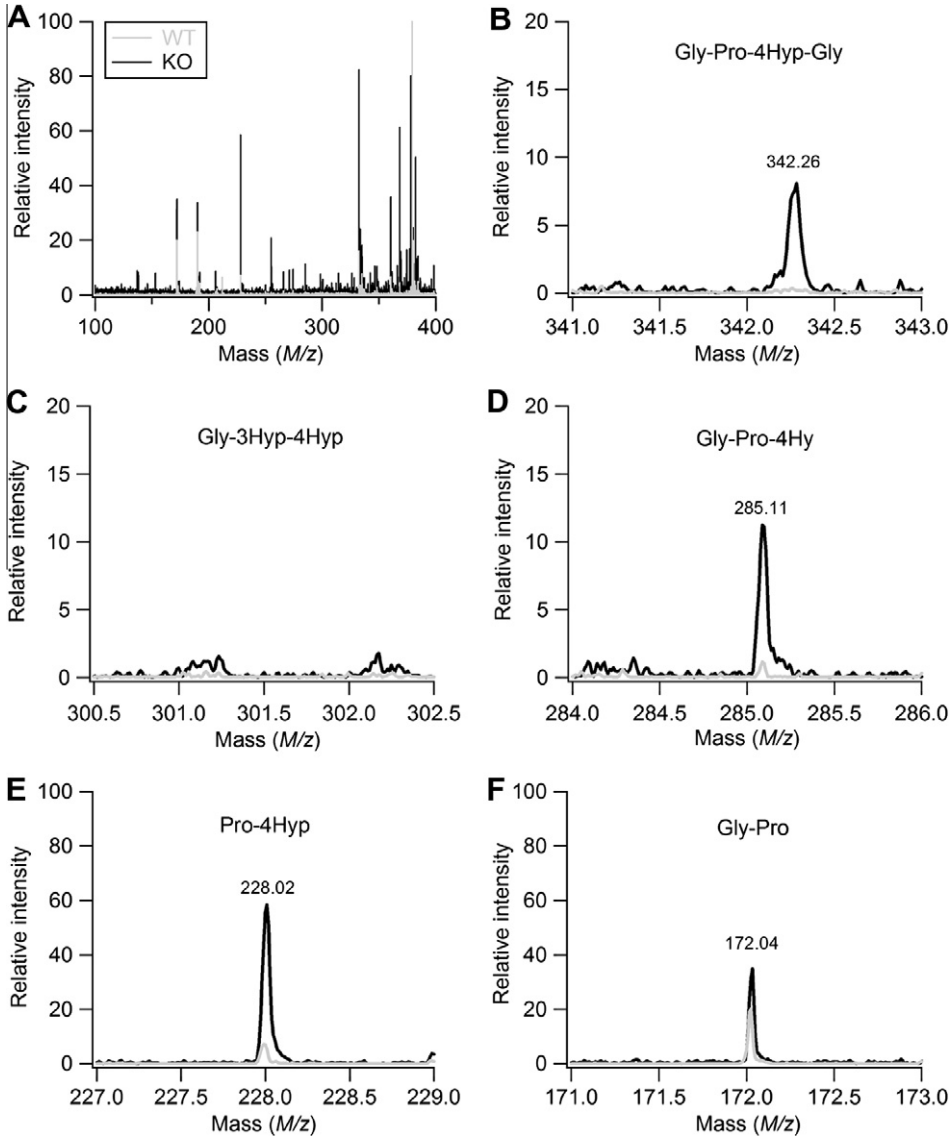

Fig. 4. Peptiduria in Xpnpep1 KO mice. (A) MALDI-TOF MS analysis of WT and Xpnpep1 urine. (B–F) MALDI-TOF mass spectrum of imino-oligopeptide in urine. The spectra were magnified to show the peaks corresponding to Gly-Pro-4Hyp-Gly (B), Gly-3Hyp-4Hyp (C), Gly-Pro-4Hyp (D), Pro-4Hyp (E) and Gly-Pro (F) in mouse urine.

ine samples from both genotypes (Fig. 4C). In addition to the tetrapeptide Gly-Pro-4Hyp-Gly, higher concentrations of Gly-Pro-4Hyp and Pro-4Hyp were detected in Xpnpep1 KO urine (Fig. 4D and E). However, dipeptide Gly-Pro was detected in the urine samples of both genotypes (Fig. 4F). Collectively, these results suggest that APP1 deficiency causes peptiduria in mice.

4. Discussion

The expression of APP1 in various organisms has been reported, but little is known about its physiological roles. In this study, we generated Xpnpep1 KO mice and examined the physiological consequences of genetic APP1 ablation in vivo. The disruption of normal APP1 function in mice results in severe developmental retardation, microcephaly, and peptiduria. To the best of our knowledge, this is the first report that provides direct evidence of such a critical role for APP1-mediated peptide metabolism in body and brain development.

This study demonstrates that APP1 deficiency in mice causes neurological defects as well as non-neurological defects. The neurological defect microcephaly was also observed in one of the APP-deficient human subjects. Deficiency of APP activity in humans causes peptiduria [18]. Massive urinary excretion of undigested imino-oligopeptides was observed in brothers with decreased APP activity. However, microcephaly was observed in a 5-month-old boy but not in his 5-year-old brother. One possible reason is that the peptiduria and neurological defects observed in patients are not linked and that the neurological phenotypes are not caused by APP deficiency. Another possibility is that APP-deficient phenotypes are expressed differently in humans during distinct developmental stages. Interestingly, the excretion of undigested peptide in the younger brother was decreased in an age-dependent manner as he grew. These results imply that APP activity is more important in the early stages of human development. It seems that there are more APP substrates during the early developmental stages. In accordance with this hypothesis, Xpnpep1 KO mice show lethality in the embryonic or neonatal stages but not in the adult stages. The third possibility is that neurological phenotype expression requires less APP activity than that required for peptiduria. It is conceivable that the almost complete loss of APP activity causes neurological defects. In our study, neurological phenotypes were observed in Xpnpep1 KO mice, possibly because of a complete loss of APP1. However, the absolute APP activity in each subject is unknown.

Although the genetic factors that cause APP deficiency in humans are currently unknown, APP1, but not APP2 or APP3, is likely to be responsible for such dysfunction, for the following reasons. First, the undigested imino-oligopeptides observed in peptiduria originate from endogenous sources, as supported by the fact that exogenously loaded peptides with penultimate proline residues do not affect the levels of excreted imino-oligopeptides [18]. This finding indicates that at least APP2, membrane-bound extracellular isoform, is unlikely to be involved in imino-oligopeptide production within this study subject. In addition, APP3 has very weak expression in the brain compared to APP1 [21], and neither microcephaly nor developmental retardation was observed in humans with an APP3 mutation [17].

According to the previous biochemical results, APP1 shows broad substrate specificity for peptides of diverse sizes ranging from dipeptides to longer peptides. Additionally, APP1 can hydrolyze many biologically active peptides, including bradykinin, and neurotransmitters, such as substance P, neuropeptide Y, and peptide YY [9,10]. Judging from the cytosolic localization of APP1, this protein is unlikely to participate in the initial degradation of neuropeptides, although the possibility has not been tested in neurons. APP1 might be involved in the final stage of peptide degradation. In lysosomes, neuropeptides with a penultimate proline can be degraded to X-pro dipeptides through the action of dipeptidyl peptidase II (DPP II; EC 3.4.14.2) and/or dipeptidyl peptidase IV (DPP IV; EC 3.4.14.5) with other proteases [22]. Because peptides with a penultimate proline are resistant to the attack of other peptidases and because any released X-pro dipeptides can easily cross the lysosomal membrane [23], APP1 effectively cleaves X-pro dipeptides into amino acids in the cytosol. In the absence of APP1 activity in Xpnpep1 KO mice, accumulated X-pro dipeptides in the neuron might cause neurodevelopmental disorders. The peptide substrates responsible for neurodevelopmental disorders in Xpnpep1 KO mice are currently unknown. More than 23 bioactive peptides with a penultimate proline residue are found in mammals, and spatially restricted signaling of each peptide can affect a distinct brain region. However, circulating peptides with a penultimate proline in their periphery are unlikely to affect the Xpnpep1 KO mouse brain because molecules larger than dipeptides rarely cross the blood–brain barrier [24]. Among the potentially fruitful avenues for future studies is an investigation of brain APP1 peptide substrates to identify those that are associated with neurodevelopmental disorders.

Considering that microcephaly and inborn metabolic errors are often associated with epilepsy or intellectual disability [25–28], there is a possibility that APP1 deficiency causes neuropsychiatric disorders in humans. Thus, the findings obtained from Xpnpep1 KO mice will be helpful in establishing a better understanding of these disorders and treating diseases associated with APP1 deficiency.

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