Review

Homocysteine Thiolactone Detoxifying Enzymes and Alzheimer's Disease

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Homocysteine Thiolactone Detoxifying Enzymes and Alzheimer's Disease

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Abstract: Elevated levels of homocysteine (Hcy) and related metabolites are associated with Alzheimer's disease (AD). Severe hyperhomocysteinemia causes neurological deficits and worsens behavioral and biochemical traits associated with AD. Although Hcy is precluded from entering the Genetic Code by proofreading mechanisms of aminoacyl-tRNA synthetases, and thus is a non-protein amino acid, it can be attached to proteins via an *N*-homocysteinylation reaction mediated by Hcy-thiolactone. Because *N*-homocysteinylation is detrimental to protein's function and biological integrity, Hcy-thiolactone detoxifying enzymes – PON1, BLMH, BPHL – have evolved. This review provides an account of the biological function of these enzymes and of consequences of their impairments leading to phenotypes characteristic of AD.

Keywords: BLMH; BPHL; PON1; homocysteine thiolactone; PHF8; mTOR signaling; autophagy; Alzheimer's disease

1. Introduction

Alzheimer's disease (AD), the most common cause of dementia, is a major health problem in aging populations [1]. AD is characterized by the extracellular accumulation of amyloid β (Aβ) and the intracellular accumulation of neurofibrillary tangles of hyperphosphorylated tau protein leading to neuronal death. Mutations in amyloid precursor protein (APP), presenilin 1 (PSEN1) and presenilin 2 (PSEN2) are responsible for the familial early-onset AD, which is relatively rare [2]. Although lifestyle and environmental factors have emerged as modulators of the susceptibility to AD [3], the causes of the most common sporadic late-onset AD are largely unknown, and no effective therapy is available. Thus, identification of novel risk factors and their mechanisms of action has important public health implications. Hyperhomocysteinemia (HHcy) is an emerging risk factor for AD [4]. However, mechanism(s) underlying the involvement of HHcy in AD are not fully understood. Specifically, it is not clear whether elevated levels of homocysteine (Hcy) itself or its downstream metabolites, such as Hcy-thiolactone (HTL) and *N*-homocysteinylated proteins, can be involved in AD.

Cystathionine β-synthase (CBS) deficiency, the most prevalent inborn error in the sulfur amino acid metabolism [5,6], is biochemically characterized by severe HHcy, i.e., severely elevated levels of Hcy and its metabolites, Hcy-thiolactone [7] and *N*-Hcy-protein [8,9]. CBS deficiency affects the central nervous system and causes severe learning and intellectual disability [5], reduced IQ [10], psychosis, obsessive-compulsive and behavior/personality disorders [11]. Accelerated brain atrophy associated with HHcy has been reported in healthy elderly individuals [12], alcoholic patients [13], and in AD patients [14] who also show upregulated brain mTOR signaling [15]. These phenotypes are also seen in an animal model of human CBS deficiency, the *Cbs*$^{-/-}$ mouse. Specifically, in the *Cbs*$^{-/-}$ mouse model, severe HHcy is accompanied by neurological impairments and cognitive deficiency characterized by attenuated problem-solving abilities, learning, short- and long-term memory [16,17]. The expression of the histone demethylase Phf8 was reduced, H4K20me1, mTOR signaling, and App were increased in brains of *Cbs*$^{-/-}$ mice compared to *Cbs*$^{+/-}$ sibling controls. Autophagy-related proteins Becn1, Atg5, and Atg7 were downregulated while neurodegeneration-related neurofilament-L (Nfl)

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and glial fibrillary acidic protein (Gfap) were upregulated in *Cbs*$^{-/-}$ brains. Treatments with Hcy-thiolactone, *N*-Hcy-protein or Hcy, or *Cbs* gene silencing by RNA interference significantly reduced Phf8 expression and increased total H4K20me1 as well as mTOR promoter-bound H4K20me1 in mouse neuroblastoma N2a and N2a-APPswe cells. This caused transcriptional mTOR upregulation, autophagy downregulation, and significantly elevated APP and Aβ levels. The *Phf8* gene silencing increased Aβ, but not APP, levels. These findings show that Phf8 regulates Aβ synthesis and suggest that neuropathy seen in mouse Cbs deficiency is mediated by Hcy metabolites, which transcriptionally dysregulate the Phf8->H4K20me1->mTOR->autophagy pathway thus increasing Aβ accumulation [18]. Phospho-Tau level was also elevated in *Cbs*$^{-/-}$ mouse brain [19].

Because *N*-homocysteinylated by Hcy-thiolactone is detrimental to protein's function and biological integrity [9,20–23], enzymes detoxifying Hcy-thiolactone have evolved: serum paraoxonase 1 (PON1) [24], cytoplasmic bleomycin hydrolase (BLMH) [25], and mitochondrial biphenyl hydrolase-like (BPHL) enzyme [26–28], all of which hydrolyze Hcy-thiolactone to Hcy. The enzymatic detoxification reaction protects proteins from *N*-homocysteinylation [24,29] because it eliminates Hcy-thiolactone, which would otherwise damage them [9,20,22,30,31].

Accumulating evidence suggests that Hcy-thiolactone hydrolyzing enzymes PON1, BLMH, and BPHL play an important role in the central nervous system. This review provides an overview of current understanding of the biological function of Hcy-thiolactone-detoxifying enzymes and of the consequences of their impairment leading to phenotypes characteristic of AD. To provide a context for the discussion of Hcy-thiolactone-detoxifying enzymes, Hcy metabolism, biogenesis, and chemical biology of Hcy-thiolactone and *N*-homocysteinylated proteins are also briefly summarized.

2. Homocysteine Metabolism

Hcy was synthesized in 1935 by the reduction with metallic sodium-ammonia [32] of the disulfide homocystine (Hcy-S-S-Hcy), obtained three years earlier in 1932 by boiling Met in sulfuric acid [33]. The article describing the first synthesis of Hcy also reported the synthesis of Hcy-thiolactone from Hcy in strongly acidic solutions [32]. Later studies clarified the role of Hcy formed as a product of Met metabolism in a reaction catalyzed by the enzyme AHCY [34] (Figure 1) and as a precursor of the sulfur amino acids Met (reaction (i)) and cysteine (reaction (ii)) [35], and of the thioester Hcy-thiolactone (reaction (iii)) [9,22] (Figure 1).

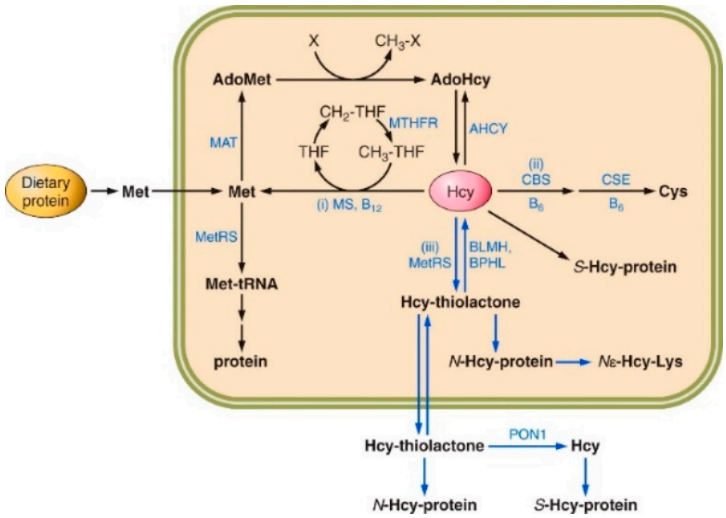

Figure 1. Schematic representation of homocysteine metabolism in humans and mice: the remethylation (i), transsulfuration (ii), and homocysteine (Hcy)-thiolactone (iii) pathways. Protein metabolism-related reactions involving Hcy are highlighted by blue arrows. The rectangle symbolizes the cell, the outside area is plasma, and the oval labelled "Dietary protein" represents the digestive tract. See text for description. AdoMet, adenosylmethionine; BPHL, biphenyl hydrolase-like; CBS, cystathionine β-synthase; MAT, Met S-adenosyltransferase; Met, methionine; MetRS, methionyl-

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tRNA synthetase; MS, Met synthase; MTHFR, methylenetetrahydrofolate reductase; THF, tetrahydrofolate. Reproduced with permission from Jakubowski [9].

In humans and other mammals, Hcy is generated from Met as a byproduct of the S-adenosylmethionine (AdoMet)-mediated methylation reactions [35]. Met, an essential amino acid supplied with protein in a diet, is released in the digestive system, taken up by epithelium, and metabolized to Hcy via the Met->AdoMet->AdoHcy->Hcy pathway in various organs (Figure 1). Hcy is then metabolized to Hcy-thiolactone by methionyl-tRNA synthetase (MetRS or MARS) [21,36,37], remethylated back to Met, or transsulfurated to Cys [35] (Figure 1). The genetic or dietary deficiencies affecting transsulfuration (CBS, CSE) or remethylation (MS, MTHFR) enzymes (Figure 1) lead to the accumulation of Hcy, Hcy-thiolactone [7,21,37,38], and *N*-Hcy-protein [8,21,37,39], and are associated with various pathologies in humans [4,9].

3. Homocysteine Thiolactone

Hcy-thiolactone, an intramolecular thioester of Hcy, was first synthesized from methionine (Met) in 1934 [40] as a byproduct of an assay for the quantification of Met in proteins [40]. The assay involved demethylation of Met by boiling with hydriodic acid, which also produced methyl iodide, whose quantification provided the basis of the assay. A more recent study showed that the digestion of L-Met with hydriodic acid yields a D,L-Hcy-thiolactone racemate [41]. Because the recovery of Hcy-thiolactone was quantitative [40], the hydriodic acid digestion provided a convenient method for the preparation of D,L-[^{35}S]Hcy-thiolactone [41], which facilitated the elucidation of Hcy-thiolactone metabolic pathways [9,20–23,37].

The enzymatic conversion of Hcy to Hcy thiolactone in an editing reaction of MARS, prevents access of Hcy to the Genetic Code [42,43], and is universal, occurring in all cell types and organisms investigated so far, from bacteria [44,45] and yeast [46], to plants [47], mice [7], and humans [21,36,37,48]. The Hcy editing reaction is the only known mechanism of Hcy-thiolactone biosynthesis [42,49,50] (Figure 1). The fundamental role of MARS in Hcy-thiolactone biosynthesis in mammalian cells has been established by showing that Chinese hamster ovary cells harboring a temperature-sensitive mutation in the gene encoding MARS are unable to synthesize Hcy-thiolactone at a non-permissive temperature [36] and that Hcy-thiolactone formation in human endothelial cells was inhibited by Met [21].

In mice, Hcy-thiolactone is quickly cleared from the body ($t_{1/2}$ = 5.1 min) [51,52], about 6-times faster than Hcy ($t_{1/2}$ = 31.8 min) [51,52], and 120-times faster than *N*-Hcy-protein ($t_{1/2}$ = 10.2 h) [51] (Figure 2). Efficient Hcy-thiolactone clearance is responsible for its relatively low levels, compared to Hcy and *N*-Hcy-protein levels in humans and mice [9].

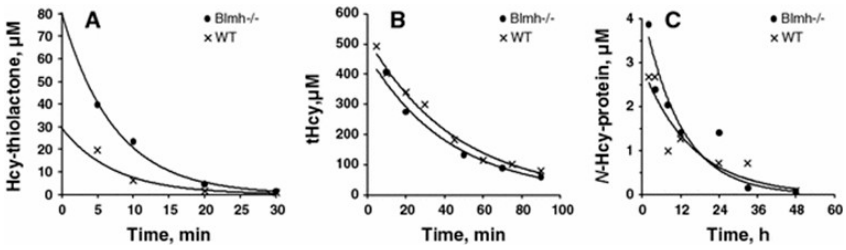

Figure 2. Kinetics of plasma Hcy-thiolactone (**A**), total Hcy (**B**), and *N*-Hcy-protein (**C**) turnover in mice. For Hcy-thiolactone (**A**) and total Hcy (**B**) turnover experiments, mice were injected i.p. with 600 nmol L-Hcy-thiolactone/g body weight. For *N*-Hcy-protein (**C**) turnover experiments, 2,850 nmol L-Hcy-thiolactone/g body weight L-Hcy-thiolactone was used. Metabolites were analyzed at indicated times post-injection and data points were fitted to an exponential equation $[A^t] = [A^0] \cdot e^{-k \cdot t}$, where k is a first-order rate constant, $[A^t]$ is metabolite concentration measured at time t, and $[A^0]$ is metabolite concentration extrapolated to time zero. Representative kinetics obtained for individual knockout *Blmh*$^{-/-}$ (•) and wild-type *Blmh*$^{+/+}$ (x) mice are shown. Reproduced with permission from Borowczyk et al. [51].

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4. *N*-Homocysteinylated Proteins

Hcy-thiolactone is harmful because of its ability to chemically modify protein lysine residues, which impairs protein structure and function [20,37], as first shown for human *N*-homocysteinylated (*N*-Hcy)-albumin [53], whose K525Hcy modification increased the protein's susceptibility to oxidation and proteolysis [53]. Two other *N*-homocysteinylated lysine residues were identified in human albumin in vivo: K137Hcy and K212Hcy [54,55]. Interestingly, *N*-homocysteinylation of K212 in mice was affected by sex: significantly more K212Hcy modification in male than in female mice [9].

Notably, albumin, a classical globular protein with predominantly α-helical secondary structures, was converted by *N*-homocysteinylation to amyloid-like aggregates with prevailing β-sheet secondary structures [56].

Subsequent studies showed that *N*-homocysteinylation of other proteins conferred on them immunogenic [57,58], atherogenic [59,60], thrombogenic [8,61,62], amyloidogenic [56], neuropathic [63–66], and oncogenic [23,67] properties [9,22,30,31].

In cell cultures, *N*-Hcy-protein biogenesis positively correlated with the concentrations of its precursors Hcy, Hcy-thiolactone, and with the MARS activity [21]. Vitamin B_{12} and folate, cofactors of Hcy-metabolizing enzymes, inhibited *N*-Hcy-protein biogenesis [68]. Methionine, which inhibits the MARS-dependent metabolic conversion of Hcy to Hcy-thiolactone, also inhibited *N*-Hcy-protein biogenesis [21,46]. The antifolate drug aminopterin, which prevents metabolic conversion of Hcy to Met, increased *N*-Hcy-protein biogenesis [37]. In humans, *N*-Hcy-protein biogenesis increased in *CBS* and *MTHFR* deficiencies [8] and was influenced by *PON1* polymorphism [29] and PON1 arylesterase activity [69]. In mice, *N*-Hcy-protein biogenesis is affected by the diet and *Cbs*, *Mthfr*, *Pcft*, *Pon1*, and *Blmh* genotypes [22].

Additional evidence supporting the mechanism of *N*-Hcy-protein biogenesis comes from the identification by mass spectrometry of specific *N*-Hcy-lysine (KHcy) residues in proteins: K525Hcy, K212Hcy, and K137Hcy in human and mouse serum albumin [53–55,61]; αK562Hcy, βK344Hcy, and γK385Hcy in human fibrinogen [61,70]; K160Hcy in mouse collagen [71]; five KHcy residues (K14Hcy, K18Hcy, K23Hcy, K27Hcy, and K56Hcy) in histone H3 from HTL-treated HEK293 T cells [72]; five KHcy residues (K32Hcy, K121Hcy, K338Hcy, K1173, and K1812) in ATR from HCT116 cells [67], K1218Hcy in dynein from rat brain [63], 304 KHcy residues in proteins from HTL-treated HeLa cells [73]; 2,525 KHcy residues in 870 different proteins from NE4C cell [66]; H3K79Hcy and other histone KHcy residues in human fetal NTD brain [74]; K411Hcy in MAP1 from rat brain [75]; K80Hcy in α-synuclein from mouse brain [64]; K182Hcy in DJ-1 from HEK293 cells [65].

5. Hcy-Thiolactone Hydrolyzing Enzymes

5.1. Paraoxonase 1

Paraoxonase 1 (PON1), named for the ability of hydrolyze the organophosphate pesticide paraoxon (Table 1), is the first enzyme that was found to use Hcy-thiolactone as a physiological substrate [24] (Table 1). PON1, a monomeric enzyme of 43 kDa molecular weight, synthesized in the liver and carried in the blood attached to a minor subclass of high-density lipoprotein (HDL) that represents 5% of total HDL [76], is present in many organs, including the brain [77]. It protects from organophosphate toxicity in agricultural workers [78] and from major adverse cardiovascular events in patients with coronary artery disease [79,80] and chronic kidney disease [81]. Low PON1 Hcy-thiolactone hydrolytic activity predicted worse long-term mortality [82]. In a general population, PON1 arylesterase activity predicted major adverse cardiovascular events [83]. In mice, Pon1 protects from atherosclerosis induced by a high-fat diet [84] or ApoE depletion [85]. Cardio protection by PON1 can be due to its apparent antioxidative function mediated by interactions of PON1 with redox response-related proteins [86,87] and its ability to detoxify Hcy-thiolactone [24,52,88], which attenuate lipid/protein peroxidation [79,84,89], and protein *N*-homocysteinylation [29,52,69].

 doi:10.20944/preprints202406.0219.v1

Table 1. Substrate specificities of human Hcy-thiolactone hydrolyzing enzymes *.

Substrate	PON1, %	BLMH, %	BPHL, %
L-Hcy-thiolactone (k_{cat}/K_m)	100 (10 $M^{-1}s^{-1}$)	100 (10^3 $M^{-1}s^{-1}$)	100 ($7.7x10^4$ $M^{-1}s^{-1}$)
D-Hcy-thiolactone	24	<1	ND
γ-Thiobutyrolactone	545	<1	<0.001
N-Acetyl-*D*, *L*-HTL	<1	<1	<0.001
L-Hse-lactone	++++	–	+++
L-Met methyl ester	<1	++	30
L-Cys methyl ester	<1	++	++
L-Lys methyl ester	ND	–	–
L-Phe ethyl ester	0	ND	16
Nε-Hcy-aminocaproate	ND	++++	ND
Val(*Nε*-Hcy-Lys)	ND	++++	ND
HcyLeuAla	ND	++++	ND
Bleomycin	ND	500	ND
Paraoxon	330	–	ND
Phenyl acetate	280,000	–	<0.001
Valacyclovir	–	ND	22

* Data for PON1 and BLMH are from refs [24,25], respectively, and for BPHL from refs [26–28]. Multiple '+' symbols indicate relative enzymatic activity for indicated substrates; a '–' symbol means no activity. Hcy, homocysteine; Hse, homoserine; ND, not determined. Reproduced with permission from Jakubowski [9].

PON1 has also been implicated in AD [90–92], which may not be unexpected given that AD has a significant vascular component [93]. For example, PON1 arylesterase activity, reflecting levels of the PON1 protein [94,95], was significantly reduced in AD and dementia patients compared to healthy controls [96–99] and negatively correlated with the extent of AD-related cognitive decline [100]. In mild cognitive impairment patients, PON1 arylesterase activity predicted global cognition, verbal episodic memory, and attention/processing speed [101]. *ApoE*$^{-/-}$*Pon1*$^{-/-}$ mice with severe carotid atherosclerosis [85], also showed markers of AD and impaired brain vasculature at 14 months, although it was not clear whether brain pathology occurred due to *ApoE*$^{-/-}$, *Pon1*$^{-/-}$, or both knockouts [102]. In a Tg2576 mouse AD model, immunohistochemical signals for Pon1 surrounded Aβ plaques in various brain regions but could not be assigned to any cell type [103].

5.1.1. Consequences of *Pon1* Gene Ablation

Pon1 gene deletion in mice diminished their Hcy-thiolactone hydrolyzing ability (Table 2), causing Hcy-thiolactone accumulation in the brain, kidney, and urine. *Pon1*$^{-/-}$ mice exhibited significantly increased neurotoxic response to Hcy-thiolactone injections compared to their *Pon1*$^{+/+}$ siblings [52] (Figure 3). *Pon1*$^{-/-}$ mice were also more susceptible to organophosphates and other neurotoxic agents [78,84] and to atherosclerosis [84,85]. Exposure to organophosphates has been linked to neurological disorders including AD, Parkinson's disease (PD), intellectual disability, attention deficit hyperactivity disorder (ADHD), autism, and other developmental neuropathies [104].

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Table 2. Hcy-thiolactone hydrolyzing activity is absent in serum from *Pon1*$^{-/-}$ mice.

Genotype	Hcy-thiolactone hydrolase activity*, %		Paraoxonase activity#, %	
	Male	Female	Male	Female
Pon1$^{-/-}$	0	0	0	0
Pon1$^{+/-}$	51	30	50	40
Pon1$^{+/+}$	100	73	100	70

* Data from Jakubowski [24], and Borowczyk et al. [52]. # Data from Shih et al. [84]. Modified from Jakubowski [9].

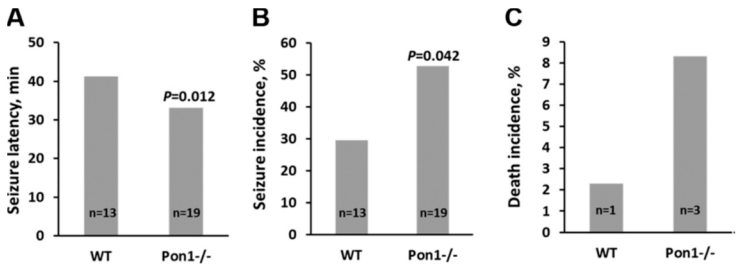

Figure 3. Increased incidence of *L*-Hcy-thiolactone-induced seizures in *Pon1*$^{-/-}$ mice relative to their *Pon1*$^{+/+}$ siblings (WT). *L*-Hcy-thiolactone was injected i.p. into *Pon1*$^{-/-}$ (n=19) and *Pon1*$^{+/+}$ (WT, n=13) mice (3.7 μmol/g body weight); the animals were monitored for 90 min. Data from Borowczyk et al. [52].

Studies of brain proteomes in *Pon1*$^{-/-}$ vs. *Pon1*$^{+/+}$ mice showed that Pon1 interacts with diverse cellular processes, such as energy metabolism, anti-oxidative defenses, cell cycle, cytoskeleton dynamics, and synaptic plasticity, that are essential for brain homeostasis [105]. The findings that Pon1 depletion influenced the expression of oxidative stress-responsive proteins associated with AD, such as Sod1, Prdx2, and DJ-1 suggests that Pon1 involvement in oxidative stress is indirect [105].

Clusterin (CLU, APOJ), involved in transport of amyloid beta (Aβ) from plasma to brain in humans (reviewed in [2]), is carried on a distinct HDL subspecies that contains three major proteins: PON1, CLU, and APOA1 [106]. Notably, levels of Clu (ApoJ) were significantly elevated in plasma of *Pon1*$^{-/-}$ vs. *Pon1*$^{+/+}$ mice [86]. Taken together, these findings suggest that PON1 plays an important role in the CNS.

5.1.2. Pon1 Depletion Downregulates Phf8, Upregulates mTOR Signaling, Inhibits Autophagy

That PON1 plays an important role in the CNS is further supported by a recent study using a new mouse model of AD, the *Pon1*$^{-/-}$5xFAD mouse, which elucidated molecular mechanism by which Pon1 maintains CNS homeostasis and protects brain from accumulation of Aβ, a hallmark of AD [107]. The study showed that Pon1 depletion, which causes accumulation of Hcy-thiolactone and *N*-Hcy-protein in mice [52], downregulated histone demethylase Phf8 and upregulated the H4K20me1 epigenetic mark in brains of *Pon1*$^{-/-}$ mice and in Pon1-silenced mouse neuroblastoma N2a-APPswe cells [107]. The depletion of Pon1 increased H4K20me1 binding to the mTOR promoter, demonstrated in mouse neuroblastoma N2a-APPswe cells, and upregulated mTOR signaling, which in turn inhibited the autophagy flux.

5.1.3. Pon1 Depletion Upregulates App and Aβ

In mouse neuroblastoma N2a-APPswe cells and in brains of *Pon1*$^{-/-}$5xFAD mice, Pon1 depletion upregulated amyloid precursor protein (App) and amyloid beta (Aβ) [107]. Treatments with *N*-Hcy-protein and Hcy-thiolactone induced similar biochemical changes App and Aβ levels in the mouse neuroblastoma cells.

These findings provide direct mechanistic evidence linking Hcy-thiolactone and *N*-Hcy-protein with dysregulated mTOR signaling and its downstream consequences such as downregulation of autophagy and upregulation of Aβ. This mechanism is further supported by findings showing that

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Phf8 depletion by RNA interference affected mTOR, autophagy, APP and Aβ, as did Pon1 depletion or treatments with Hcy-thiolactone or *N*-Hcy-protein. These findings also suggest that Pon1 is a negative regulator of mTOR signaling by controlling levels of Hcy metabolites that affect binding of H4K20me1 at the mTOR promotor and define a neuroprotective mechanism by which Pon1 protects from amyloidogenic App processing to Aβ in mouse brain [107].

5.1.4. Pon1 Interacts with App but Phf8 Does Not

Although depletion of Pon1 downregulated Phf8 and upregulated APP in brains of *Pon1*$^{-/-}$5xFAD mice, Phf8 depletion did not change APP level [107], suggesting that Pon1 interacts with APP in the mouse brain while Phf8 does not. The nature of the Pon1-APP interaction, whether it is direct or indirect, remains to be elucidated.

Pon1 depletion downregulated Phf8 and upregulated Aβ in brains of *Pon1*$^{-/-}$5xFAD mice and in mouse neuroblastoma N2a-APPswe cells. In contrast, Phf8 depletion upregulated Aβ, although it did not affect APP level [107]. This suggests that two pathways are involved in Aβ generation in Pon1-depleted mouse brain and neural cells. One pathway involves Hcy-thiolactone and *N*-Hcy-protein metabolites, which upregulate APP, while another pathway, mediated by Phf8, H4K20me1, and mTOR, involves impaired Aβ clearance due to downregulated autophagy (Figure 4).

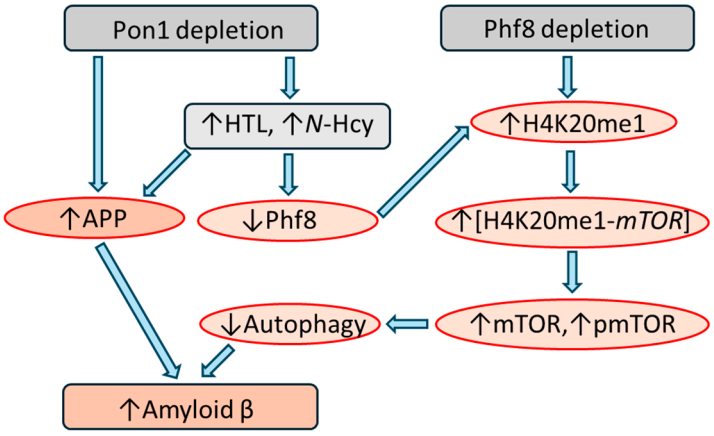

Figure 4. Hypothetical pathways leading to Aβ generation in *Pon1*$^{-/-}$5xFAD mice. Up and down arrows show direction of changes. Blmh, bleomycin hydrolase; Hcy, homocysteine; HTL, Hcy-thiolactone; APP, amyloid beta precursor protein; mTOR, mammalian target of rapamycin; pmTOR, phospho-mTOR; Phf8, Plant Homeodomain Finger protein 8. [H4K20me1-*mTOR*] represents H4K20me1 bound at the *mTOR* promoter.

5.1.5. Similar Effects of Pon1 Depletion and Hcy-Thiolactone/N-Hcy-Protein on Pathways Leading to Aβ

Interestingly, Pon1 depletion induced changes in the Phf8->H4K20me1->mTOR->autophagy pathway in *Pon1*$^{-/-}$5xFAD mouse brain and in Pon1-silenced neuroblastoma cells that mimicked the changes induced by HHcy in *Pon1*$^{+/-}$5xFAD mouse brain and in Hcy-thiolactone or *N*-Hcy-protein treated mouse neuroblastoma N2a-APPswe cells [107]. Pon1 depletion or HHcy similarly increased accumulation of Aβ in the brain. Earlier work has shown that biochemical outcomes of Pon1 depletion and HHcy were identical: HHcy elevated Hcy-thiolactone and *N*-Hcy-protein [39] as did Pon1 depletion [52,69]. Pon1 depletion by RNA interference or treatments with Hcy-thiolactone or *N*-Hcy-protein similarly elevated Aβ in mouse neuroblastoma cells. Taken together, these findings suggest that increased accumulation of Aβ in Pon1-depleted brain is mediated by effects of Hcy metabolites on mTOR signaling and autophagy. These findings also suggest that Pon1 is a negative regulator of mTOR signaling by controlling Hcy-related metabolite levels that influence the extent of H4K20me1 binding at the mTOR promoter.

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5.2. Bleomycin Hydrolase

Human BLMH [108,109], named for the ability to hydrolyze the anticancer drug bleomycin, is the second enzyme that was found to use Hcy-thiolactone as a physiological substrate [25] (Table 2). BLMH, a cytoplasmic enzyme expressed in various organs, has a quaternary structure like the 20 S proteasome, and is a member of the self-compartmentalizing cysteine proteases family [110]. In addition to being studied in relation to Hcy toxicity [25,111] and Alzheimer's disease [112–115], BLMH was also studied in the field of protein turnover [116,117], cancer therapy [109,118], and keratinization disorders [119,120]. The I443V polymorphic site located in the C-terminal domain important for the activity of the human BLMH is associated with a risk of AD in some [113,121] but not all studies [122–124].

A cytoplasmic Hcy-thiolactone hydrolyzing activity was originally purified from human placenta and identified by proteomic and biochemical analyses as BLMH [25]. Substrate specificity studies showed that the human BLMH exhibits absolute stereo-specificity for *L*-Hcy-thiolactone, the preferred natural substrate, and does not hydrolyze *D*-Hcy-thiolactone (Table 1). Methyl esters of sulfur-containing amino acids such as *L*-Cys and *L*-Met were also hydrolyzed, while *D*-Met methyl ester was not. *L*-homoserine lactone, γ-thiobutyrolactone, and other *L*-amino acids, were not hydrolyzed by human BLMH [25].

Recombinant human and yeast BLMH, expressed in *E. coli*, exhibit Hcy-thiolactone hydrolyzing activity like that of the corresponding native enzymes. Active site mutation C73A in human BLMH and H369A in yeast BLMH inactivate their Hcy-thiolactone hydrolyzing activity [25].

5.2.1. Consequences of *Blmh* Gene Ablation

In mice, the deletion of the *Blmh* gene [125] diminished the animals ability to detoxify Hcy-thiolactone, which led to its accumulation in the brain, kidney, and urine [51], and resulted in several brain-related phenotypes such as astrogliosis and behavioral changes [126], increased neurotoxic response to Hcy-thiolactone injections [51] (Figure 5), in addition to skin-related (tail dermatitis [125]) and immune response-related phenotypes (impaired antigen presentation [127]).

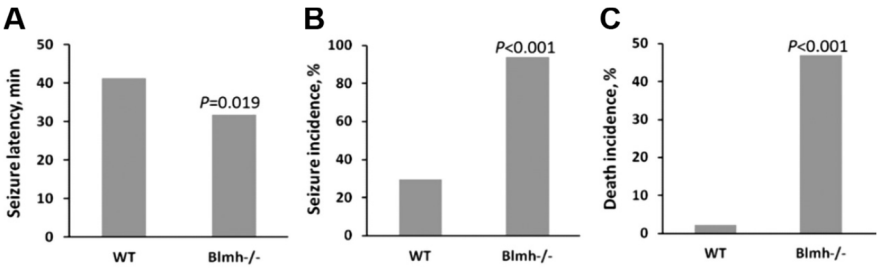

Figure 5. Increased incidence of *L*-Hcy-thiolactone-induced seizures in *Blmh1*$^{-/-}$ mice relative to their *Blmh1*$^{+/+}$ littermates (WT). *L*-Hcy-thiolactone was injected i.p. into *Blmh1*$^{-/-}$ (n = 32) and *Blmh1*$^{+/+}$ (WT, n=44) mice (3.7 μmol/g body weight); the animals were monitored for 90 min. Data from Borowczyk et al. [51].

In brains of AD patients, the Hcy-thiolactonase and aminopeptidase activities of BLMH were significantly decreased compared to control brains, suggesting that the attenuated BLMH activity contributes to the pathology of AD [111]. Serum BLMH level was significantly reduced in Parkinsons disease (PD) patients who responded to the therapeutic deep brain stimulation [128], a treatment recommended for advanced stages of PD [129]. Levels of BLMH in extracellular vesicles from the cerebrospinal fluid were significantly lower in amyotrophic lateral sclerosis patients compared with healthy controls [130]. Proteomic studies of *Blmh*$^{-/-}$ mouse brain showed that Blmh affects various cellular processes, which are important for brain homeostasis, including synaptic plasticity, cytoskeleton dynamics, cell cycle, energy metabolism, and antioxidant defenses [131]. Taken together, these findings suggest that Blmh plays an important role in the CNS.

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5.2.2. Blmh Depletion Downregulates Phf8, Upregulates mTOR Signaling, Inhibits Autophagy

To elucidate molecular mechanism by which Blmh maintains CNS homeostasis and protects brain from the accumulation of Aβ, a hallmark of AD, a recent study examined biochemical and behavioral traits related to AD in a new mouse model, the *Blmh1*$^{-/-}$5xFAD mouse [132]. 5xFAD mice overexpress the K670N/M671L (Swedish), I716V (Florida), and V717I (London) mutations in human APP(695), and M146L and L286V mutations in human PS1 associated with familial early-onset AD and accumulate elevated levels of Aβ42 beginning around 2 months of age [133].

The study showed that Blmh depletion, which causes accumulation of Hcy-thiolactone and *N*-Hcy-protein in mice [51], downregulated histone demethylase Phf8 and upregulated the H4K20me1 epigenetic mark in brains of *Blmh*$^{-/-}$ and *Blmh*$^{-/-}$5xFAD mice [132]. These findings were recapitulated in Blmh-silenced mouse neuroblastoma N2a-APPswe cells that harbor a human APP transgene with the K670N and M671L Swedish mutations associated with familial early-onset AD [134]. Blmh depletion increased H4K20me1 binding to the mTOR promoter (demonstrated in N2a-APPswe cells) and upregulated mTOR signaling, which in turn inhibited the autophagy flux in N2a-APPswe cells and in brains of *Blmh*$^{-/-}$5xFAD mice.

5.2.3. Blmh Depletion Upregulates App and Aβ and Worsens Cognitive and Neuromotor Deficits

Blmh depletion upregulated App and Aβ in mouse neuroblastoma cells and in *Blmh*$^{-/-}$5xFAD mouse brains [132]. Treatments with *N*-Hcy-protein and Hcy-thiolactone induced similar biochemical changes in mouse neuroblastoma cells. These biochemical changes were associated with cognitive and neuromotor deficits in *Blmh*$^{-/-}$ and *Blmh*$^{-/-}$5xFAD mice. For example, one-year-old *Blmh*$^{-/-}$5xFAD mice scored worse compared to *Blmh*$^{+/+}$5xFAD animals in the ovel object recognition test, indicating impaired memory, and in the hindlimb and cylinder tests, indicating sensorimotor impairments. Four-month-old *Blmh*$^{-/-}$ mice, which did not accumulate Aβ, showed similar memory and sensorimotor impairments compared to *Blmh*$^{+/+}$ animals. These findings show that the absence of the Blmh protein causes memory and sensorimotor impairments independently of the Aβ-producing transgene [51].

Neurological impairments seen in *Blmh*$^{-/-}$ and *Blmh*$^{-/-}$5xFAD mice are likely to be caused, at least partly, by Phf8 depletion, which does occur in *Blmh*$^{-/-}$ brains [132]. That Phf8 depletion could account for the neurological deficits in *Blmh*$^{-/-}$ and *Blmh*$^{-/-}$5xFAD mice, is supported by findings showing that PHF8 depletion in humans causes neurological impairments such as intellectual disability, autism spectrum disorder, and attention deficit hyperactivity disorder [135,136] and that *Phf8*$^{-/-}$ mice also show similar neuropathies [137].

5.2.4. Treatments with Hcy-Thiolactone or *N*-Hcy-Protein Mimicked the Effects of Blmh Depletion

Notably, treatments with Hcy-thiolactone or *N*-Hcy-protein mimicked the effects of Blmh depletion by siRNA treatments in the mouse neuroblastoma cells [132]. For example, Hcy-thiolactone, *N*-Hcy-protein, or Blmh depletion inhibited Phf8 expression, elevated total H4K20me1 level, increased H4K20me1 bound at the mTOR promoter, upregulated mTOR signaling, and impaired autophagy. These findings suggest that Blmh is a negative regulator of mTOR signaling by controlling Hcy-related metabolite levels that influence the extent of H4K20me1 binding at the mTOR promoter [132].

Phf8 is also a mediator directly linking Hcy-thiolactone and *N*-Hcy-protein with dysregulated mTOR signaling and its downstream outcomes such as impaired autophagy flux and upregulated Aβ accumulation, thus supplying a plausible mechanism explaining neuropathy induced by Blmh deficiency [132] (Figure 6) and explaining an association of HHcy with Alzheimer's disease [4]. This function of Phf8 is further supported by experiments showing that *Phf8* gene silencing had the same impact on mTOR, autophagy, and Aβ as did *Blmh* gene silencing or the treatments with Hcy-thiolactone or *N*-Hcy-protein [132].

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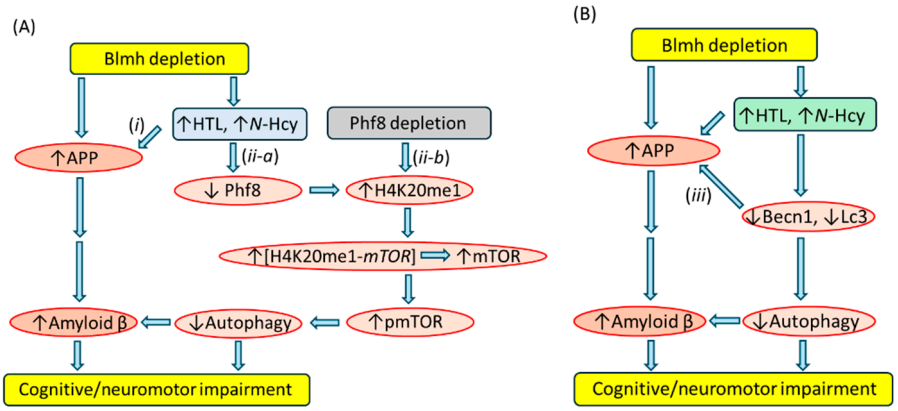

Figure 6. Hypothetical pathways leading to Aβ upregulation in *Blmh*$^{-/-}$5xFAD mice. Panel (A) illustrates the APP (*i*) and Phf8 (*ii-a*) pathways. Panel (B) highlights the interaction (*iii*) between autophagy (Becn1) and APP pathways. Up and down arrows show direction of changes. Blmh, bleomycin hydrolase; Hcy, homocysteine; HTL, Hcy-thiolactone; APP, amyloid beta precursor protein; mTOR, mammalian target of rapamycin; pmTOR, phospho-mTOR; Phf8, Plant Homeodomain Finger protein 8. [H4K20me1-*mTOR*] represents H4K20me1 bound at the *mTOR* promoter. Modified from Witucki et al. [132].

5.2.5. Blmh Interacts with App, but Phf8 Does Not

Importantly, *Blmh* gene deletion upregulated App in *Blmh*$^{-/-}$ in *Blmh*$^{-/-}$5xFAD mice as did *Blmh* gene silencing in mouse neuroblastoma N2a-APPswe cells [132]. However, silencing the *Phf8* gene had no effect on App expression, suggesting that the Blmh interacts with App in the CNS while Phf8 does not. The interaction Blmh-App interaction is most likely direct, as suggested by other investigators who found that human BLMH interacts with APP in vitro and that overexpressed BLMH processed human APP to Aβ in the 293-HEK and CHO cells [138]. Another report showed that rat Blmh has the ability to hydrolyze Aβ40 and Aβ42 *in vitro*, with fibrillar Aβ forms being more resistant than nonfibrillar Aβ [115]. Another possibility is that BLMH can regulate mTOR expression via binding to the *mTOR* promoter, supported by findings that BLMH can bind to DNA [139,140]. Further studies are needed to clarify the mechanism underlying the regulation of APP by BLMH.

Although Blmh depletion downregulated Phf8 and upregulated APP and Aβ, Phf8 depletion upregulated Aβ but not APP. These findings suggest that three pathways contribute to Aβ upregulation in Blmh-depleted mouse brain (Figure 6) [132]. In the first pathway (*i*, Figure 6A), Hcy metabolites upregulate APP (independently of Phf8), which leads to Aβ upregulation in Blmh-depleted or Hcy-thiolactone/*N*-Hcy-protein-treated mouse brain cells. In the second pathway, (*ii-a*, Figure 6A), Hcy metabolites downregulate Phf8, which upregulates mTOR signaling and thereby reduces autophagy flux resulting in Aβ upregulation due to impaired clearance. Direct depletion of Phf8 by RNA interference, independently of Hcy metabolites, also starts a similar pathway mediated by mTOR (*ii-b*, Figure 6A) that results in Aβ accumulation due to impaired autophagy. These pathways remain to be verified in future studies by testing effects of Phf8 overexpression or mTOR downregulation (by pharmacological inhibition using rapamycin or by RNA interference) on APP and Aβ accumulation in *Blmh*-depleted cells.

5.2.6. Becn1 Interacts with App

The findings that APP upregulation was accompanied by downregulation of Becn1, a protein with a central role in autophagy initiation, in the *Blmh*$^{-/-}$5xFAD mouse brain and in mouse neuroblastoma cells suggest that a third pathway, involving an interaction between Bcln1 and APP, contributes to Aβ upregulation [132]. In this pathway, Becn1 is a negative regulator of APP expression and processing (*iii*, Figure 6B). This conclusion is supported by prior findings showing that level of Becn1 is significantly reduced in human AD brains compared with non-AD controls, and that reduction of Becn1 level in transgenic APP-overexpressing *APP*$^{+}$*Becn*$^{+/-}$ mice increased Aβ

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accumulation in neuronal cells [141]. Becn1 was also reported to regulate APP processing and turnover. Depletion of Bcln1 by siRNA in rat neuroblastoma B103/hAPPwt cells expressing human APP transgene elevated APP, Lc3, and Aβ, while overexpression of Becn1 reduced APP level [142]. The involvement of autophagy in APP and Aβ accumulation Blmh-depleted cells needs to be confirmed in future studies, e.g., by enhancing autophagy (*e.g.*, with TAT-Beclin1), which should rescue APP and Aβ accumulation in these cells.

5.2.7. Similar Effects of Blmh Depletion and Hcy-Thiolactone/N-Hcy-Protein on Pathways Leading to Aβ

Interestingly, *Blmh* gene deletion or HHcy induced by a high Met diet led to similar changes in the Phf8->H4K20me1->mTOR->autophagy pathway and Aβ accumulation [132]. These findings are consistent with the fact that the *Blmh* gene deletion and high Met diet lead to the same biochemical outcome: upregulation of Hcy-thiolactone and *N*-Hcy-protein levels [39,51]. Indeed, treatments of mouse neuroblastoma cells with individual metabolites that accumulate in HHcy, Hcy-thiolactone or *N*-Hcy-protein, recapitulated changes in the Phf8->H4K20me1->mTOR->autophagy pathway and Aβ accumulation seen in Blmh-depleted or Met-supplemented HHcy wild type mice [132]. These findings also suggest that dysregulation of Hcy metabolism in general would affect the Phf8->H4K20me1->mTOR->autophagy pathway in the CNS. Indeed, Hcy metabolites inhibit autophagy, elevate Aβ, and induce neuropathy by dysregulating Phf8/H4K20me1-dependent epigenetic regulation of mTOR in cystathionine β-synthase-deficient mice and Cbs-silenced mouse neuroblastoma cells [18].

Blmh deficiency or HHcy induced by a high Met diet elevated level of methylated histone H4K20me1 via downregulation of the histone demethylase Phf8 in mouse brain [132]. HHcy is also known to affect DNA and protein methylation via *S*-adenosylhomocysteine (AdoHcy, an inhibitor of cellular AdoMet-dependent methylation reactions), which underlies the pathology of HHcy-associated human disease (reviewed in ref. [143]). However, possible inhibition of H4K20 histone methylase by AdoHcy would have an opposing effect, *i.e.*, it would *reduce* H4K20me1 level. The findings linking Blmh with the status of the histone H4K20me1 methylation, are reminiscent of findings showing that Pon1 deletion in mice elevated the H4K20me1 methylation level via downregulation of Phf8 [107] (Figure 4). Thus, these two Hcy-thiolactone-detoxifying enzymes exert similar effects on H4K20me1 levels. Although there is no evidence that Blmh or Pon1 are linked to DNA methylation, these findings provide the first evidence that Blmh and Pon1 influence histone methylation.

5.3. Biphenyl Hydrolase-Like Enzyme

Biphenyl hydrolase-like (BPHL) enzyme, also called valacyclovir hydrolase, is the third enzyme shown to use Hcy-thiolactone as a natural substrate [26–28]. It is a 32 kDa mitochondrial protein highly expressed in human liver and kidney [144,145]. BPHL hydrolyzes and activates the antiviral prodrug esters valacyclovir and valganciclovir, used in the treatment of herpes simplex, herpes zoster (shingles), and herpes B [146]. However, valacyclovir was rapidly hydrolyzed to acyclovir in *Bphl*$^{-/-}$ mice, which shows that BPHL is not obligatory for the conversion of valacyclovir to acyclovir [147].

First cloned from the breast carcinoma cells and expressed in *E. coli*, BPHL, a member of the alpha/beta hydrolase fold family, is a serine hydrolase distantly related to other members of the serine hydrolase family [144,145]. The *BPHL* gene is located on human chromosome 6p25 in a locus with other serine hydoxylases.

Crystallographic studies showed that human BPHL has the catalytic triad S122-D227-H255, a serine hydrolase consensus sequence GSXSG, and a unique binding mode and the specificity for esters of α-amino acids [148]. The α-amino acid specificity, including the ability of BPHL to hydrolyze L-Met methyl ester shared with the Hcy-thiolactone-hydrolyzing enzyme BLMH [25], suggested that BPHL could also hydrolyze Hcy-thiolactone. Indeed, this prediction, was substantiated by conference reports published in 2010-2011 [26,27] and a report published in a 2014 PlosOne article [28].

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BPHL, BLMH, and PON1 differ in their specificities towards non-physiological substrates for which they have been originally named and in catalytic efficiencies towards Hcy-thiolactone (Table 1). Catalytic efficiency of BPHL in the Hcy-thiolactone hydrolytic reaction is higher than that of BLMH or PON1, suggesting that BPHL can have a significant contribution to Hcy-thiolactone detoxification in vivo [28].

5.3.1. Consequences of *Bphl* Ablation

A recent study found that *BPHL* gene is overexpressed in lung cancer and promotes lung carcinogenesis and that downregulation of BPHL expression by RNA interference inhibited tumor growth and metastasis by impairing the progression of cell cycle and inducing apoptosis in A549, NCI-H1975, and NCI-H-1299 human lung carcinoma cell lines [149]. Deletion of the *Bphl* gene in mice decreased circulating creatinine levels in males, suggesting a kidney function defect (http://www.informatics.jax.org/allele/allgenoviews/MGI:5548556).

A recent report has shown that the deletion of *Bphl* gene in mice significantly attenuated Hcy-thiolactone turnover in vivo [150], similar to the impairment of Hcy-thiolactone turnover in *Blmh*$^{-/-}$ mice [51]. Notably, silencing the *Bphl* gene by RNA interference in mouse neuroblastoma N2a-APPswe cells caused changes in the Phf8->H4K20me1->mTOR->autophagy pathway and APP/Aβ levels characteristic of AD [150], similar to the changes seen in mouse neuroblastoma N2a-APPswe cells in which *Pon1* [107] or *Blmh* [132] was silenced by RNA interference.

6. Conclusions

Hcy-thiolactone, a product of an error-correcting reaction during protein biosynthesis, is generated in the human body when Hcy is selected in place of methionine by methionyl-tRNA synthetase. Hcy-thiolactone is a chemically reactive thioester metabolite that modifies protein lysine residues in a process called *N*-homocysteinylation. The modification causes protein damage/aggregation, a hallmark of many diseases, including Alzheimer's. Hcy-thiolactone-detoxifying enzymes—serum paraoxonase PON1 carried in the circulation on high-density lipoprotein, cytoplasmic bleomycin hydrolase BLMH, and mitochondrial biphenyl hydrolase-like enzyme BPLH—protect the human body proteins from Hcy-thiolactone/N-homocysteinylation-associated damage. Depletion of any of these enzymes elevates Hcy-tiolactone and *N*-Hcy-protein, which dysregulate the Phf8->H4K20me1->mTOR->autophagy pathway and upregulate APP, causing Aβ accumulation, a hallmark of Alzheimer's disease.

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