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Impact of Vitamin D on Amyloid Precursor Protein Processing and Amyloid-β Peptide Degradation in Alzheimer's Disease

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Key Words

Amyloid precursor protein · Amyloid-β peptide degradation · Alzheimer's disease

Abstract

Ninety percent of the elderly population has a vitamin D hypovitaminosis, and several lines of evidence suggest that there might be a potential causal link between Alzheimer's disease (AD) and a non-sufficient supply with vitamin D. However, the mechanisms linking AD to vitamin D have not been completely understood. The aim of our study is to elucidate the impact of 25(OH) vitamin D_3 on amyloid precursor protein processing in mice and N2A cells utilizing very moderate and physiological vitamin D hypovitaminosis in the range of 20–30% compared to wild-type mice. We found that already under such mild conditions, amyloid-β peptide (Aβ) is significantly increased, which is caused by an increased β-secretase activity and BACE1 protein level. Additionally, neprilysin (NEP) expression is downregulated resulting in a decreased NEP activity further enhancing the effect of decreased vitamin D on the Aβ level. In line with the in vivo findings, corresponding effects were found with N2A cells supplemented with 25(OH) vitamin D_3. Our results further strengthen the link between AD and vitamin D_3 and suggest that supplementation of vitamin D_3 might have a beneficial effect in AD prevention.

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Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disease of the central nervous system and is characterized by degeneration of neurons in multiple brain regions. One of the characteristic histopathological hallmarks are extracellular β-amyloid plaques [1]. Major components of the β-amyloid deposits are hydrophobic amyloid-β peptides (Aβ), generated by proteolytic amyloidogenic processing of the amyloid precursor protein (APP) [2], by β-secretase BACE1, followed by γ-secretase cleavage. Beside the production of Aβ by the amyloidogenic processing of APP, Aβ levels are also determined by

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its degradation, mediated mainly by neprilysin (NEP) and insulin-degrading enzyme (IDE) [3].

Vitamin D_3 is a secosteroid derived from 7-dehydrocholesterol by UV exposure, transported by the vitamin-D-binding protein in the blood and converted to 25(OH) vitamin D by cytochrome P450 2R1 in the liver. 25(OH) vitamin D is transported to the target tissue where it is hydroxylated to $1,25(OH)_2$ vitamin D_3 by 1-α-hydroxylase [4]. Recently, it has been shown that the receptor for 25(OH) vitamin D_3 (vitamin D receptor) is expressed in the brain, suggesting a role of vitamin D in brain function [5].

Several studies indicate a link between vitamin D and AD. However, the underlying mechanism is not clearly understood. Vitamin D hypovitaminosis affects up to 90% of the elderly population [6] and has been reported to be associated with decreased cognitive function [7], whereas vitamin D supplementation has been reported to increase cognitive performance [8]. Antioxidant effects, a decrease in hyperparathyroidism, anti-ischemic properties, anti-inflammatory actions and a link to acetylcholine neurotransmitter levels were suggested as underlying mechanisms [9]. However, other studies show no link between vitamin D and cognitive performance or AD [10].

Here, we investigate the effect of vitamin D hypovitaminosis in mice on APP processing and Aβ degradation and further analyze the mechanism of Aβ clearance in N2A cells as a neuronal cell line.

Material and Methods

All chemicals used in this study were purchased from Sigma-Aldrich (Taufkirchen, Germany), if not stated otherwise.

Mice and Cell Culture

N2A cells were incubated with 100 nM 25(OH) vitamin D or ethanol as solvent control over a period of 4 weeks in 5% FCS/DMEM (Sigma-Aldrich) under cell culture conditions as described in Grimm et al. [15]. For determination of the Aβ level in the supernatant of incubated N2A cells, the cells were transiently transfected with human APP695 using Lipofectamine 2000 (Invitrogen, Darmstadt, Germany) in accordance with the manufacturer's guidelines. Media were collected 48 h after transfection, with intermediate change of media after 24 h, and used for further analysis.

Female C57BL/6 mice (Charles River, Sulzfeld, Germany), aged 6 weeks at the beginning of the feeding experiment, were kept in a controlled environment, with a temperature at 20–22°C, 50–60% humidity and lights on between 7 AM and 7 PM. Food and water were freely available throughout the study. All procedures concerning the mice were carried out in accordance with German laws governing the use of laboratory animals. Mice were fed for 6–9 months with C1000 (control) and C1017 (vitamin-D-deficient) diet (Altromin, Lange, Germany). Both diets were isocaloric and identical with respect to their protein, carbohydrate, fiber and mineral content.

Secretase Activity Assays

β-Secretase and γ-secretase assays have been performed as described in Grimm et al. [15] and the α-secretase assay in accordance with Grimm et al. [16].

RT-PCR Experiments

RT-PCR experiments have been done as published in Grimm et al. [17] using the following primers: cathepsin B: forward AAGCTGTGTGGCACTGTCCT, reverse ATTGTTCCCGTGCATCAAAG; endothelin-converting enzyme 1: forward GATCAAGGTCGGGAGTACGA, reverse GTATTGCTGCACCATGCACT; endothelin-converting enzyme 2: forward GGTGCTGAGTGAGGTAAGCC, reverse GACCAGTCATAACGGGATTGA; IDE: forward GCTACGTGCAGAAGGACCTC, reverse TGGACGTATAGCCTCGTGGT; angiotensin-I-converting enzyme: forward CCTGAGTTCTGGAACAAGTCG, reverse TTGATCCTGAAGTCCTTGCC; matrix metallopeptidase 2: forward GACAAGTGGTCCGCGTAAAG, reverse ATCACTGCGACCAGTGTCTG; matrix metallopeptidase 9: forward CATGCACTGGGCTTAGATCA, reverse GCTTAGAGCCACGACCATACA; membrane metalloendopeptidase: forward TGAACTTTGCCCAGGTGTG, reverse GCAAAGTCCCAATGATCCTG; plasminogen: forward CCTCAGTTCCACCAGAGGAG, reverse AGGACTGGCACTTCTTCCCT; β-actin: forward CCTAGGCACCAGGGTGTGAT; reverse TCTCCATGTCGTCCCAGTTG.

Western Blot Experiments

Western blot (WB) experiments have been performed utilizing the following antibodies: anti-Bace1 PC526 (Calbiochem, Darmstadt, Germany), anti-ADAM10 (Merck, Darmstadt, Germany, 422751), anti-nicastrin N1660 (Sigma-Aldrich) and anti-IDE ST1120 (Calbiochem). Aβ immunoprecipitation and WB using the W02 antibody have been done as described in Grimm et al. [18].

Aβ Degradation

Confluent N2A cells were cultivated in 0.1% FCS/DMEM (Sigma-Aldrich) for 16 h. After removal, fresh media including 0.5 μg/ml human Aβ40 was added for 6 h. The remaining Aβ in the cell culture supernatant of these cells was detected by WB using the W02 antibody as described above.

NEP Activity Assay

NEP activity measurement was performed as published in Miners et al. [19], with minor modifications, utilizing the antibody AF1182 (R&D Systems, Minneapolis, Minn., USA).

Aβ Elisa

Aβ was determined in mouse brain lysates utilizing Elisa kits (KMB3481 and KMB3441; Invitrogen), as described by the manufacturer's protocol. Samples were lysed in 50 mM Tris/HCl, pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1× protease inhibitor cocktail containing AEBSF [4-(2-aminoethyl)-benzensulfonylfluoride; Calbiochem] and adjusted to 225 μg protein.

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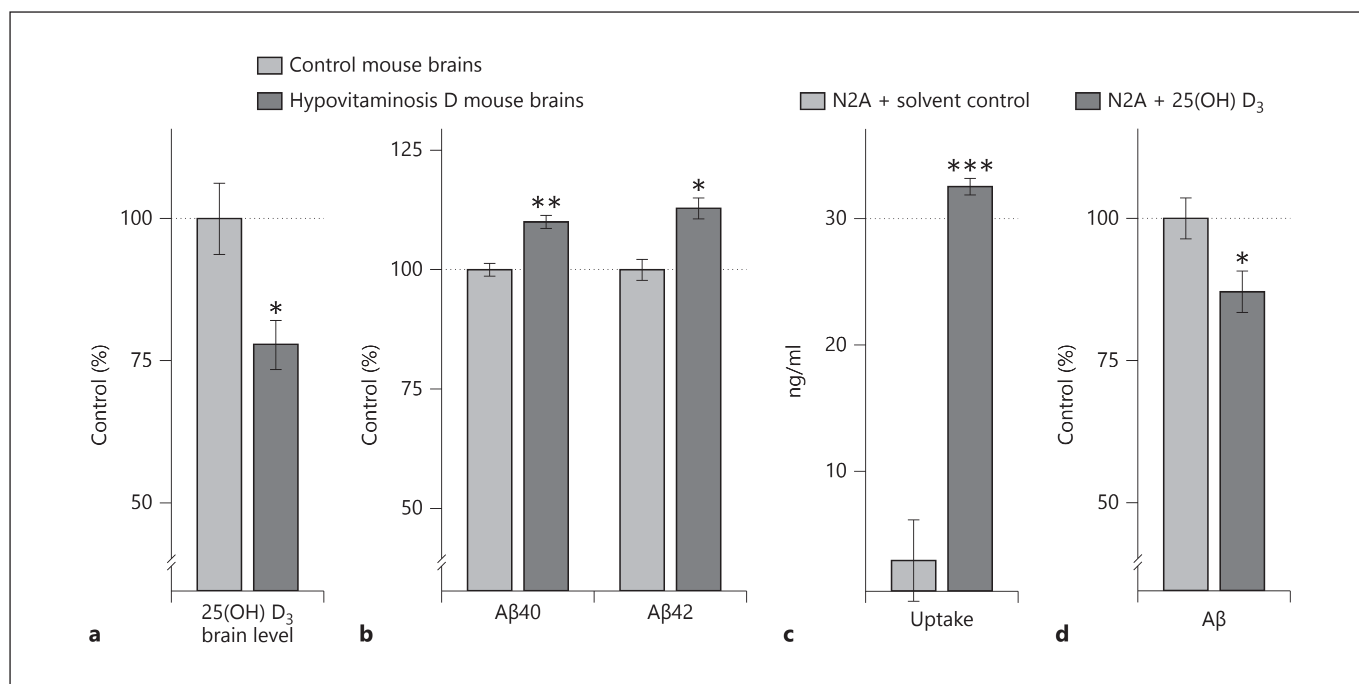

Fig. 1. Effect of vitamin D on Aβ level. Error bars represent the standard deviation of the mean. Asterisks show the statistical significance calculated by unpaired Student's t test. **a** 25(OH) vitamin D_3 [25(OH) D_3] level of wild-type and hypovitaminosis D_3 mouse brains (n = 4; * $p \leq 0.05$). **b** Effect of hypovitaminosis D_3 on Aβ40 and Aβ42 levels in 9 vitamin-D_3-deficient and 9 control mouse brains (* $p \leq 0.05$; ** $p \leq 0.01$). **c** Uptake control of 25(OH) vitamin D_3 [25(OH) D_3] in incubated N2A cells (n = 3; *** $p \leq 0.001$). **d** Total Aβ level of 25(OH) vitamin D_3 [25(OH) D_3] in incubated N2A cells (n = 5; * $p \leq 0.05$).

25(OH) Vitamin D Elisa

The 25(OH) vitamin D level in homogenates of incubated N2A cells and mouse brain tissue were determined by the 25(OH) vitamin D Elisa kit (EQ6411-9601; Euroimmun, Lübeck, Germany), according to the manufacturer's protocol, with slight modifications. For mouse brain analysis, 25(OH) vitamin D was measured after lipid extraction of 5 mg protein according to Bligh and Dyer [20].

Data Analysis

WBs were quantified by densiometric analysis using ImageJ software. All quantified data represent an average of at least three independent experiments as described in the figure legend. In figures, the error bars represent the standard deviation of the mean. Statistical significance was determined by unpaired, two-tailed Student's t test; significance was set at * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

Results

Aβ Production

The 25(OH) vitamin D level of hypovitaminosis D mice showed a 23 ± 4.33% (p = 0.027) reduction compared to the control group (fig. 1a). Both Aβ40 (10 ± 1.4%; p = 0.006) and Aβ42 (12.8 ± 2.18%; p = 0.014) levels were significantly elevated (fig. 1b), whereas the Aβ40/42 ratio showed no significant change (2.5 ± 3.0%; p = 0.57). To substantiate the effect observed in vivo, N2A cells were incubated with 25(OH) vitamin D_3. After 4 weeks of treatment, an increase in 25(OH) vitamin D_3 from 2.45 ± 3.28 ng/ml in untreated to 32.56 ± 0.67 ng/ml ($p < 0.001$) in incubated cells (fig. 1c) was observed. In the presence of 25(OH) vitamin D_3, a 13 ± 3.63% (p = 0.036) decrease in the total Aβ level (fig. 1d) was found. It should be considered that N2A cells were cultivated at slightly reduced FCS concentrations (5% v/v). 25(OH) vitamin D_3 is transported in the blood via binding to the vitamin-D-binding protein. Therefore, the FCS can be assumed to be the major source of 25(OH) vitamin D in cell culture. A reduction in FCS is used to simulate a hypovitaminosis D status. Supplementation of 25(OH) vitamin D under these conditions results in decreased Aβ production. However, this does not necessarily implicate a decrease in Aβ production upon 25(OH) vitamin D supplementation under non-hypovitaminosis but normal conditions.

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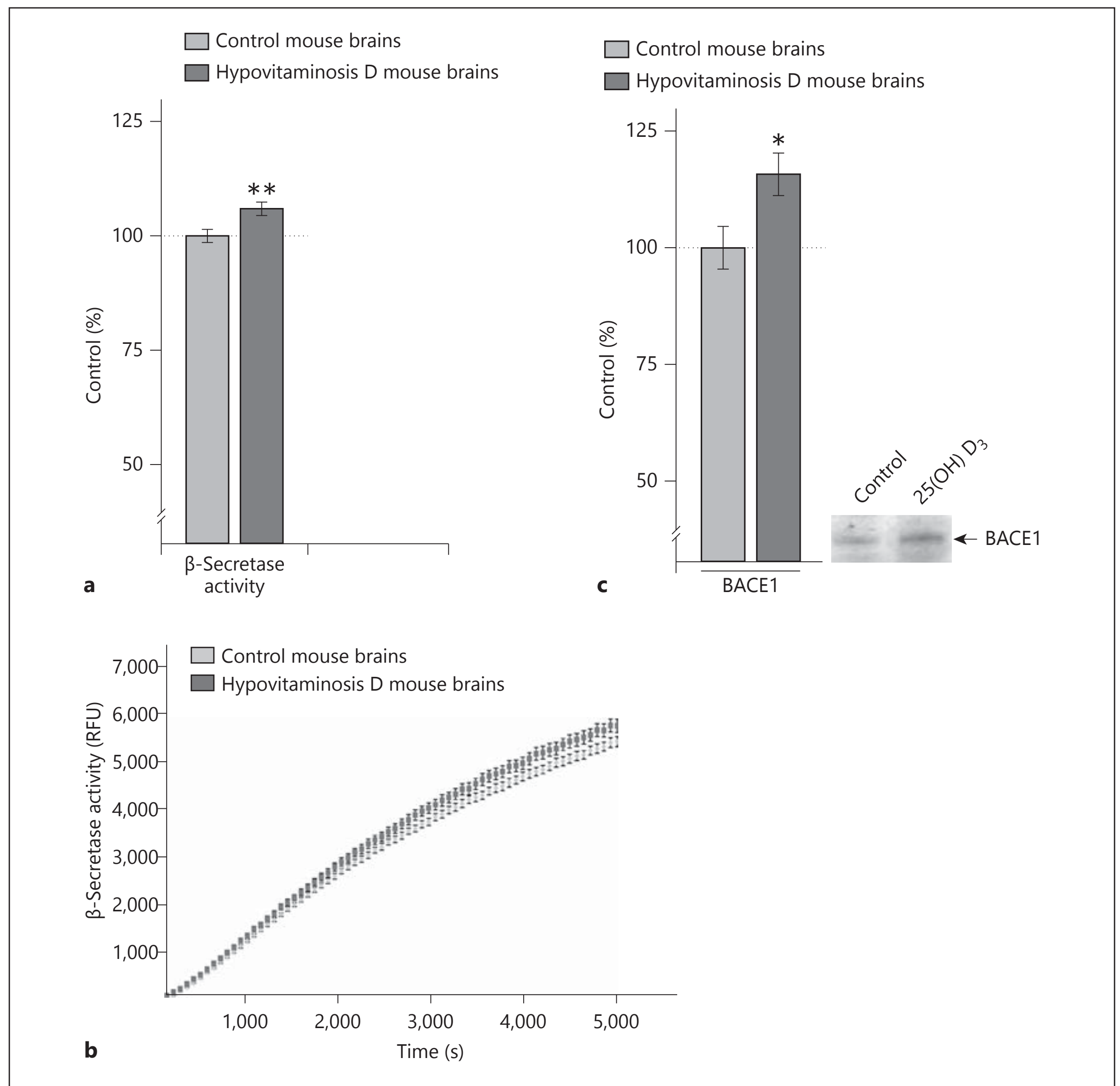

Fig. 2. Vitamin D influences β-secretase. The statistical significance was calculated as described for figure 1. **a**, **b** Effect of hypovitaminosis D_3 in 15 vitamin-D-deficient and 15 control mouse brains on BACE1 activity. **a** Quantification of the slope (** $p \leq 0.01$). **b** β-Secretase kinetic. RFU = Relative fluorescence unit. **c** BACE1 protein level in hypovitaminosis D_3 mouse brains (n = 8; * $p \leq 0.05$). 25(OH) D_3 = 25(OH) vitamin D_3.

Secretases

To clarify the underlying mechanism of an elevated Aβ production in hypovitaminosis D mice, the secretase activities were measured. β-Secretase activity was slightly but significantly elevated (6 ± 1.44%; p = 0.007; fig. 2a, b) whereas γ- and α-secretase showed no significant change (data not shown). The elevated β-secretase activity was reflected by an increased BACE1 protein level (16 ± 4.56%; p = 0.028; fig. 2c) and, in line with the unchanged γ- and α-secretase activity, ADAM10 and nicastrin protein levels were unchanged (data not shown). These results suggest that an increased β-secretase activity caused by an elevated BACE1 protein level contributes to the higher Aβ level found in hypovitaminosis D mice. However, the effect on β-secretase activity was apparently lower than the effect on the Aβ level, pointing towards one or more additional contributing mechanisms.

Aβ Clearance

To evaluate whether decreased Aβ degradation might be such an additional contributing mechanism, NEP activity in hypovitaminosis D mice was measured. Indeed,

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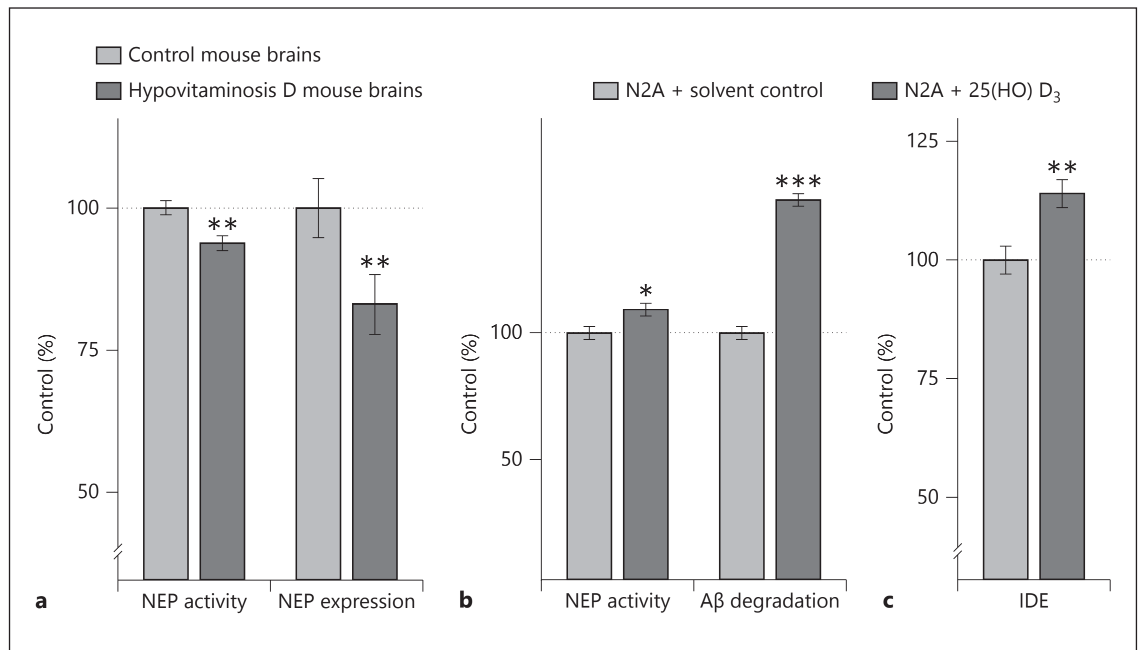

Fig. 3. Effect of vitamin D on Aβ degradation. The statistical significance was calculated as described for figure 1. **a** Effect of hypovitaminosis D_3 in mouse brains on NEP activity (n = 10; ** $p \leq 0.01$) and NEP expression (n = 8; ** $p \leq 0.01$). **b** NEP activity (n = 5; * $p \leq 0.05$) and total Aβ degradation of 25(OH) vitamin D_3 [25(OH) D_3] in incubated N2A cells (n = 10; *** $p \leq 0.001$). **c** IDE protein level of 25(OH) vitamin D_3 [25(OH) D_3] incubated N2A cells (n = 5; ** $p \leq 0.01$).

Table 1. Changed expression level of Aβ-degrading enzymes after 25(OH) vitamin D_3 incubation in N2A cells (n ≥6) measured by quantitative real-time PCR

Degrading enzyme	Mean, %	SEM, %	p value
Cathepsin B	128.7	8.0	0.005
Endothelin-converting enzyme 1	104.7	25.9	0.857
Endothelin-converting enzyme 2	101.8	9.6	0.849
Insulin-degrading enzyme	105.3	9.1	0.574
Angiotensin-I-converting enzyme	156.1	19.5	0.011
Matrix metallopeptidase 2	132.6	22.5	0.162
Matrix metallopeptidase 9	140.8	27.2	0.153
Membrane metalloendopeptidase	186.5	35.8	0.028
Plasminogen	170.7	24.7	0.009

The statistical significance was calculated as described for figure 1.

NEP activity was reduced (7 ± 1.3%; p = 0.003; fig. 3a). In line with the decreased activity, gene expression is downregulated (17 ± 5.24%; p = 0.009; fig. 3a). 25(OH) vitamin D supplementation in N2A cells resulted in 9 ± 2.6% increased NEP activity (p = 0.032; fig. 3b). Please note that in cell culture experiments total Aβ degradation was highly increased (54 ± 2.44%; p < 0.001; fig. 3b) suggesting that other Aβ-degrading enzymes might be involved. Accordingly, the IDE protein level was elevated (14 ± 2.9%; p = 0.009), and the expression of several other known Aβ-degrading enzymes was increased as well (table 1). However, other than for NEP, the effect of additional Aβ-degrading enzymes was not confirmed in vivo, making further studies necessary.

Discussion

In this study, we aimed to examine the effect of mild vitamin D hypovitaminosis in experimental conditions which might be similar to the situation typical for humans with vitamin D hypovitaminosis [11]. Therefore, mice with an average 23% 25(OH) vitamin D decrease were analyzed. Additionally, we decided not to use transgenic but wild-type mice, avoiding a potential influence of overexpressed and mutagenized APP or presenilin. Although the conditions used here were mild, significant changes could be observed in the Aβ level. Assuming that a 23% reduction in the vitamin D_3 level results in a 10–12% increase in the Aβ level suggests a change of 0.5% in

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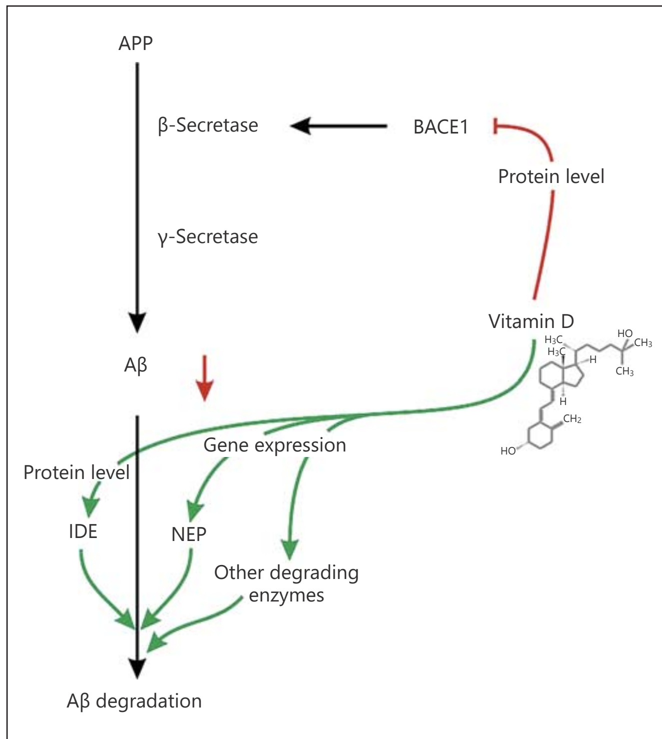

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Fig. 4. Schematic overview of the vitamin-D-mediated effects on APP processing and Aβ degradation.

the Aβ level per 1% change in the 25(OH) vitamin D_3 level. Similar results were already obtained by Yu et al. [12], showing that the Aβ level in transgenic mice was altered dependent on vitamin D. Mice which were fed with a vitamin-D-enriched diet showed an increase of 75% in the vitamin D level (38.53 compared to 21.98 ng/ml vitamin D) followed by a 48% (Aβ40) or 46% (Aβ42) Aβ reduction. In agreement with our results, that study suggests a similar 0.7% decrease in the Aβ level per 1% increase in the vitamin D level. Moreover, they observed a vitamin-D-dependent reduction in β-C-terminal fragment, a product of APP derived by β-secretase cleavage, in line with our observation of vitamin-D-dependent alterations in β-secretase activity and BACE1 protein level. Besides the change in Aβ level – in the study by Yu et al. [12], a change in the plaque load was observed – one might hypothesize that the increased Aβ degradation found in our study might also contribute to this finding. The reduced NEP activity in vitamin-D-reduced mice can be explained by decreased NEP expression. Notably, the nuclear vitamin D receptor has been suggested to be involved in transcriptional regulation in neurons and glia cells [13], which could also be involved in the upregulation of NEP by vitamin D. Similar effects have been shown by Nalivaeva et al. [3] in osteoblast-like cells, where NEP was affected by 1,25(OH) vitamin D_3 in the concentration range of 0.01–1 μM for 72 h.

Summing up, we showed that under physiologically relevant hypovitaminosis conditions, Aβ is increased, and in neuronal cell lines, Aβ can be decreased by adding 25(OH) vitamin D. Vitamin D hypovitaminosis increases β-secretase activity and the BACE1 protein level. 25(OH) vitamin D supplementation increases Aβ degradation, largely due to NEP activity caused by increased NEP expression (fig. 4). Therefore, our results clearly support the idea that vitamin D hypovitaminosis is a risk factor for AD and that supplementation of vitamin D in the elderly population with hypovitaminosis might be beneficial.

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