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Profiling of Alzheimer´s disease related genes in mild to moderate vitamin D hypovitaminosis

Running title: Alzheimer´s Disease genes are regulated by vitamin D

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Keywords: Alzheimer's disease, vitamin D hypovitaminosis, vitamin D-receptor, gene expression

Abstract: A vast majority of the elder population shows a mild to moderate vitamin D deficiency. Besides the well-known function of vitamin D, vitamin D-receptor is also expressed in brain and is discussed to regulate several genes. However very little is known whether genes are regulated, associated with Alzheimer´s disease (AD). Here we investigate 117 genes, known to be affected in AD, in mouse brain samples with a mild vitamin D hypovitaminosis comparable to the vitamin D status of the elderly population (20-30% deficiency). The 117 genes include two positive controls, *Nep* and *Park7*, already known to be affected both by AD and vitamin D hypovitaminosis. The 25 most promising candidates were verified in a second independent mouse-cohort resulting in eleven genes further evaluated against three additional housekeeping genes. Three of the remaining eight significantly altered genes are involved in APP-homeostasis (*Snca*, *Nep*, *Psmb5*), and each one gene in oxidative stress (*Park7*), inflammation (*Casp4*), lipid metabolism (*Abca1*), signal transduction (*Gnb5*) and in neurogenesis (*Plat*). Our results tighten the link of vitamin D and AD and underline that vitamin D influences several genes also in brain, highlighting that not only a strong link to AD but also to other neurodegenerative diseases might exist.

Keywords: Alzheimer’s disease, vitamin D hypovitaminosis, vitamin D-receptor, gene expression

1. Introduction

Alzheimer´s Disease (AD), a progressive and irreversible neurodegenerative disorder, is characterized among other factors by increased accumulation of the neurotoxic Amyloid-β (Aβ) peptide, a product of the sequential proteolytic processing of the amyloid precursor protein (APP). The amyloidogenic cleavage of APP results besides Aβ in the release of the APP intracellular domain (AICD), which is discussed to regulate gene transcription [1-8]. Furthermore, it has been shown that increased Aβ formation leads to elevated lipid peroxidation and subsequent oxidative stress, associated with high levels of reactive oxygen species (ROS) [9] and an increased proinflammatory cytokine activation [10] emphasizing the multifactorial character of this metabolic disease.

Besides several lipids that influence Aβ homeostasis, it has recently been shown that lipophilic vitamins affect molecular mechanisms involved in AD pathogenesis [11]. Especially vitamin D_3, a secosteroid derived from 7-dehydrocholesterol by UV-exposure, is discussed to have beneficial properties in respect to AD. Biological activities of the active calcitriol ($1,25(OH)_2D_3$) can be attributed to binding interactions with the vitamin D receptor (VDR), which is a ligand-activated transcription factor. After binding to vitamin D_3 the VDR undergoes a conformational change and interacts with another transcription factor called the retinoid X receptor (RXR). This active VDR-RXR-heterodimer binds to vitamin D response elements in the DNA of target genes [12]. Besides being expressed in the intestine, bone, skin and a variety of other tissues [13, 14], the VDR is also present in the human brain, suggesting a role of vitamin D in brain function [15, 16].

85% of the elderly population has a vitamin D hypovitaminosis [17], amongst others because of an age-related decreased capacity of their skin to produce vitamin D_3 and because of a homebound sunlight-deprived lifestyle [18, 19]. Several clinical studies suggest a potential link between AD and a non-sufficient supply with vitamin D [20, 21].

Taking into consideration that vitamin D affects gene expression of several genes e.g. via VDR mediated pathways and a strong link between vitamin D and AD in clinical studies exists, the rationale of our study was to investigate whether genes known to play a role in AD are regulated by vitamin D hypovitaminosis. Identifying new genes crucial in the AD pathology which are altered by vitamin D deficiency would provide a potential causal link between vitamin D hypovitaminosis and AD. Therefore, we selected 117 genes, known to be involved in AD pathology, and examined if their gene expression is changed by a decreased vitamin D level. In our study we used a mouse model having a 20-30% vitamin D hypovitaminosis and analysed the gene expression in mouse brain samples compared to non-deficient control mice [22]. A 20-30% vitamin D hypovitaminosis was chosen as these conditions reflect the situation of the vitamin D status of 85% of the elderly, also termed mild to moderate hypovitaminosis D in the human population [17, 23]. The 117 genes include two genes, which are already known to be regulated by vitamin D

deficiency. These genes act as a positive control to clarify whether a 20-30% vitamin D hypovitaminosis in mice utilized in this study is sufficient to detect these known changes in gene expression in brain.
Out of these 117 AD-related genes the 25 top candidates were evaluated in a second independent mouse cohort. The remaining significantly changed target genes were normalized against three additional housekeeping genes. The design of the study is summarized in figure 1.

2. Materials and Methods

2.1 Vitamin D deficient mice

The used brain samples were obtained from female wild-type C57BL/6 mice (Charles River, Sulzfeld, Germany) with a nutrition related vitamin D deficit. Mice were maintained in a controlled environment (temperature: 20-22 °C, humidity: 50-60%, 12-hour dark/light cycle) with freely available food and water. In this study, two mouse populations were analysed consisting of four and three wild type and vitamin D deficient mice, respectively. All animal experiments were approved by the "Landesamt für Soziales, Gesundheit und Verbraucherschutz of the State of Saarland" (reference number 17/2011) following the national guidelines for animal treatment. The feeding experiments started at an age of six weeks and mice were fed with C1000 (control) or C1017 (vitamin-D-deficient) diet (Altromin, Lange, Germany) for > six months. In respect to protein, carbohydrate, fiber and mineral content, both isocaloric diets were identical. The 25(OH) vitamin D level in mouse brain tissue was determined as described earlier and mice fed with vitamin D deficient diet show a 23% reduced vitamin D level compared to control fed mice [22]. This corresponds to the mild to moderate vitamin D hypovitaminosis reported in the elderly population [17, 23, 24].

2.2 Gene expression analysis

At the end of the feeding experiment, mouse brains were removed, washed twice in ice-cold 0.9% sodium chloride and shock-frosted in liquid nitrogen. To isolate RNA, brain samples were slowly defrosted on ice and the TRIzol Reagent (Thermo Fisher Scientific) was used as described by the manufacturer. According to the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) 2 µg RNA were applied to generate cDNA. Gene expression analysis was performed by real-time polymerase-chain-reaction (RT-PCR) with the Fast SYBR Green Master Mix (Applied Biosystems, Foster City, California, USA) on a PikoReal Real-Time PCR System (Thermo Fisher Scientific). The used primers (Eurofins MWG Operon, Eberberg, Germany) are listed in table 1. Four housekeeping genes (β-*actin*, *Polr2f, Mprip* and *Atp5b*) were used for a part of the samples to exclude RT efficiency differences randomly. Gene expression was normalized to the expression of the housekeeping gene and changes were calculated using the $2^{-(\Delta\Delta Ct)}$ method.

Table 1. Primers used for gene expression analysis by RT-PCR.

gene	primer forward	primer reverse
A2m	GACGCAGGGACACAAGAAAG	TCCATTGGGCAGAATGGTAT
Aass	ACGGGAGGGTCCATAGATTT	GGAGCCTTCAACACTGTCGT
Abca1	CATGAAGGTTGCTGTGGATG	TTGACATGGTGGTGGTCTTC
Acat1	GCTGCAGGAAGTAAGATGCC	GGAAGGATCCAATGGGAGTT
Acat2	CCCGTCATGGGAGTAACCT	ATCGTTCATTCCTGATGCGT
Acat3	TGGGCAAGCTGAAACCTTAC	CATAAGGACCACAGCAGCAG
Ache	GCAGCAATATGTGAGCCTGA	AGTATCGGTGGCGCTGAG
Ace	CCTGAGTTCTGGAACAAGTCG	TTGATCCTGAAGTCCTTGCC
Actb	CCTAGGCACCAGGGTGTGAT	TCTCCATGTCGTCCCAGTTG
Adam9	AAGCTGCCTGCTTAACATCC	ACCTCACACTCCTTCGCTGT
Adam17	TGACATCAAGTACCGAACGC	GAGTCAGGCTCACCAACCAC
Als2	CTCTTCAATGATGCCCTGGT	CCATTCACGCTACCAGCTTC
Apba1	AGCCAATGACTGAGGTGGAC	GGAAATGGTCCTCAGAGGGT
Apba3	GTAGGGAGGTTTGCATCCAG	AGCAGGTTGGCAATGACC
Apbb1	TGCATGAGATCTGCTCCAAG	TTCCACTTGGAAAGGGACAT
Apbb2	ATCGTGAACATCCGAGTGTG	GTCACATCGAAACACATGGC
Aph1a	ACAAGCTCCTTAAGAAGGCAGA	CCGAAGGACAGACCAGAAAC
Aph1b	GCTGTTCAGGCTCGCATATT	AGAAACATAGGCCAACAGTCG
Aph1c	CTCATCGCTGGTGCTTTCTT	AGAAACATACGCCAACAGTCG
Apoa1	GCCAACAGCTGAACCTGAAT	CAGAAGTCCCGAGTCAATGG
Apoe	CTGAACCGCTTCTGGGATTA	GTGCCGTCAGTTCTTGTGTG
App	CCGTTGCCTAGTTGGTGAGT	GTGCCAGTGAAGATGGGTCT
Appbp1	CTGCTGCTGTAGGCAATCAC	ACCACTCGAAGAAATGCAGAA
Atp5b	GGATCTGCTGGCCCCATAC	CTTTCCAACGCCAGCACCT
Bace1	ACATTGCTGCCATCACTGAA	TCCAAAGAAGGGCTCCAAAGA
Bace2	GCATGCTGGACAAATTCTGA	TGTAGAGCTGTGGGAGAATGG
Bche	ATTTCCCTGGAGTGAGCAGA	CCAAAGCGTCACGGTAGACT
Bdnf	AGGACGCGGACTTGTACACT	CATAGACATGTTTGCGGCAT
Casp3	ACGCGCACAAGCTAGAATTT	CTTTGCGTGGAAAGTGGAGT
Casp4	TGGTGGTGAAAGAGGAGCTT	GCCATGAGACATTAGCACCA
Cat	AACTGGGATCTTGTGGGAAA	TGTGGGTTTCTCTTCTGGCT
Cdc2	AGGAAGAAGGAGTGCCCAGT	TACAGCCTGGAGTCCTGCAT
Cdk5	GTCCATCGACATGTGGTCAG	GTGTCCCTAGCAGTCGGAAG
Cdkl1	GCATGCTCAAGCAACTCAAG	TCGCAGTACTCGAACACCAG
Chat	ACTCCTGAGGCTCTGGCTTT	GTACTCAGTTTGGGCCTGGA
Clu	CTGTGTGCAAGGAGATCCG	TGGTTGAACAGTCCACAGACA
Ctsb	AAGCTGTGTGGCACTGTCCT	ATTGTTCCCGTGCATCAAAG
Ctsg	ACATCCAAATGCGAGAAAGG	CAGCTGCAGAAGCATGATGT
Ctsl	AAGGGTTGTGTGACTCCTGTG	TGCCGGTCTTAAGGAACATC

Duox1	AAGGGCTGAAGATGTGGATG	AGGCCAGAAATCTTGCATGT
Ece1	GATCAAGGTCGGGAGTACGA	GTATTGCTGCACCATGCACT
Ece2	GGTGCTGAGTGAGGTAAGCC	GACCAGTCATAACGGGATTGA
Ep300	CTTCCACTCCGCTTTCTCAG	GCTGCTTCTCAGGAATGGTC
Epx	GTCCAGATCATCACCTACCGA	CCACATTGGAGCAATACCCT
Ercc2	TACCCGGAGCAGTTCTCCTA	GGACACTGTCTTCCCAGTGC
Ercc6	CAGTCCAGGCAGATGCTACA	TGTAATCAGCGGCTGTCTTG
Ern1	GTGATCACTCCCAGCACAGA	CATGGTGTCCTATGAGAAGCC
Gab1	AGCCTGAACCTAACAGAACCC	GAGGAAGCAGGAGTCTGGTG
Gap43	TTGCTGATGGTGTGGAGAAG	AAGGTGCATCTCCTGCCTT
Gnao1	GACAAGGAGAGGAAGACGGA	AGTCGCATCATGGCAGAAA
Gnb1	TGTTTCCTTCTCCAAGAGTGG	CCAGCTAAGACACCTGCTCTG
Gnb2	ACTAACAAGGTCCACGCCAT	AGCAGATGTTGTCCAAACCC
Gnb4	TTGTGATGCATCCTCAAAGC	CTCGGGAAGAAACTGACAGC
Gnb5	GAAGACCAGAAGGACCCTCA	ATCACCTTCCCATCCTGTGA
Gpx1	GTTCGGACACCAGGAGAATG	TTCTCACCATTCACTTCGCA
Gpx2	GGGCTGTGCTGATTGAGAAT	GACAGTTCTCCTGATGTCCGA
Gpx3	GGCTTTGTGCCTAATTTCCA	GTGAGCCCAGGAGTTCTGC
Gpx5	ATGCACTCCAGGAGGATCTG	CCTGGACGAACATACTTGAGC
Gpx6	TGAGTATGGAGCCAACACCA	TGTGTTCAGCTCAGGGTACG
Gpx7	ACTTCAAGGCGGTCAACATC	CTGTGAAGCCACATTCGCTA
Gsk3a	GGAGCCCAATGTGTCCTACA	GCTCAGCAAGTACACAGCCA
Gsk3b	GACTTTGGAAGTGCAAAGCAG	CGTGTAATCAGTGGCTCCAA
Hadh2	GGCCAACGTGGAGTTATCAT	CAGTGTCATGCCCACTATGC
Hdac1	TCTGACCATCAAAGGACACG	AACATTCCGGATGGTGTAGC
Hmgcr	ATCGAGCCACGACCTAATGA	TAAGCTGGGATATGCTTGGC
I de	GCTACGTGCAGAAGGACCTC	TGGACGTATAGCCTCGTGGT
Idh1	GCTTCATCTGGGCCTGTAAG	TGGACAAATCAGCACACTGG
Il1a	CCCATGATCTGGAAGAGACC	TGACAAACTTCTGCCTGACG
Ins	AGAGGCTCTCTACCTGGTGTGT	CCTCCCAGCTCCAGTTGTT
Insr	TCTTCGAGAACGGATCGAGT	TTGGCTGTCCTTTGGATACC
Lpl	GATGCCCTACAAAGTGTTCCA	CCACTGTGCCGTACAGAGAA
Lrp1	CAGCTCACTGTGAAGGCAAG	GGTACAGTCCTTGTCGCCAT
Lrp6	GGCAGCCAAATGCTACAAAT	TGGGCAAGCACACTGATAAA
Map2	TGGCTCTCTAAAGAACATCCG	CAGGTACGTGGTGAGCATTG
Mapt	TCAGGTCGAAGATTGGCTCT	CACACTTGGACTGGACGTTG
Mmp2	GACAAGTGGTCCGCGTAAAG	ATCACTGCGACCAGTGTCTG
Mmp9	CATGCACTGGGCTTAGATCA	GCTTAGAGCCACGACCATACA
Mpo	CTCAAGATCCCACCCAATGA	TTGCGAATGGTGATGTTGTT
Mpp4	AAGTGCTGTGCCACATACCA	TCCGTACATGAGGCTTTCAA
Mprip	GGCTGGCTAACCAAGCAGTA	TCTAGGTCAGCTGCCTCCTC

Ncstn	TGCTCTATGGGTTCCTGGTT	CGGCGATGTAGTGTTGAAGA
Nep	ATGGAGACCTCGTTGACTGG	TTCCATTGAGATGCTGTCCA
Nqo1	GCCGATTCAGAGTGGCAT	CATCCTTCCAGGATCTGCAT
Nudt15	TTTGGAATTCGGTGAGACCT	AAGAATTTACCACGGAGGCA
Park7	GCCATCTGTGCAGGTCCTA	GCGGCTCTCTGAGTAGCTGTA
Pkp4	CAAACACTGGTTCAGCCATC	CGCCTGTGCTGGTAACATAA
Plat	GCTGAGTGCATCAACTGGAA	CTGGGTTTCTGCAGTAATTGTG
Plau	CCAGAAGAACAAGGGAGGAA	TTTGGGAGTTGAATGAAGCA
Plg	GGTGGGAATACTGCAACCTG	GCAGTCTGTCTCAGAGTCGCT
Polr2f	AAGCGGATCACCACTCCTTA	TGAGCAAAGGGTCTGTCTCC
Ppp1r15b	TGAATCAGACGTGGAACAGG	CGTCTGAATCGTGGCTGTAA
Prdx1	CACCCAAGAAACAAGGAGGA	CTTCATCAGCCTTTAAGACTCCA
Prdx2	TAGCGACCATGCTGAGGACT	TATTGATCCACGCCAGGTG
Prdx6	GGGCAGGAACTTTGATGAGA	GCTCTCTCCCTTCTTCCAGTC
Prkaa1	TGTTCCAGCAGATCCTTTCC	TTGAAAGACCAAAGTCGGCTA
Prkaa2	ATGCCCAGATGAACGCTAAG	ACCTGCATACAGCCTTCCTG
Prkca	GGCGGATTTATCTGAAGGCT	ATAAGGATCCGAAAGCCCAT
Prkcb1	GAGATCTGGGATTGGGACCT	AACTTGAACCAGCCATCCAC
Prkcd	GGGACACCATCTTCCAGAAA	AAACTGCCACAGTGGTCACA
Prkce	AGCTTTGGCAAGGTCATGTT	GTGCAGTCCACATCATCGTC
Prkcg	GTTCCGTCTGCACAGCTACA	CTCGCAACAGGAACATTTCA
Prkc1	CACCCTCAAACTGGATTTGC	TTTGGTTTAAAGGGTGGAACC
Prkcq	GCCTGAACAAGCAGGGTTAC	TATTGATTGCGGATCCTGTG
Prkcz	ATAGACTGGGACCTGCTGGA	TGGTGAACTGCGTGTCAAAG
Prnp	CGAGACCGATGTGAAGATGA	CTGGATCTTCTCCCGTCGTA
Psen1	ACCCGGAGGAAAGAGGAGTA	TGGTTGTGTTCCAGTCTCCA
Psen2	TCATGCTATTTCGTGCCTGTC	GTGTAGATGAGCTGCCCGTT
Psenen2	ATCTTGGTGGATTTGCGTTC	CCTTTGATTTGGCTCTGCTC
Psmb5	CAGATCTGCTGGACTTGGGT	AGAAACTTGAAGGCCAGGGT
Serpina3a	GCCCAGGATGCTAGATGAAC	CAGTGATCCCAGACAGGTCA
Snca	GGAGTGACAACAGTGGCTGA	GCTCCCTCCACTGTCTTCTG
Sncb	CAGACCTGAAGCCAGAGGAG	CTCTGGCTCGTATTCCTGGT
Sod1	CGTACAATGGTGGTCCATGA	AATCCCAATCACTCCACAGG
Tpo	CCCATACAGCTTCCCTCAAA	CCAAAGAGAGCACCTTGGTC
Txnip	GCAGCAGGTCTGGTCTGAG	TAGCAAGGAGGAGCTTCTGG
Txnrd2	GGCACCTTTGACACTGTCCT	CCTGGGCATCCACAATAATC
Ubqln1	CACCGATATCCAGGAGCCTA	GCTGAGTCCCTTCTGCTGAG
Ucp3	AAGACCCGATACATGAACGC	GGGCACAAATCCTTTGTAGAAG
Uqcrc1	GTGGTGGAGTGCACCTGTC	CCAGCAGCCCAGTATCAGAG
Uqcrc2	CAAAGGAAGTCACCAGCCTT	GCATTGATAACCTCTCCAGCA
Xpa	TGAACCACTTTGATCTGCCA	AGCGCTGGCTCTCTCTTCT

2.3 Data Analysis

If not otherwise indicated all quantified data represent an average of at least three independent experiments. Error bars represent standard error of the mean. Statistical significance was calculated by two-tailed Student´s *t* test and significance was set at $p \leq 0.05$.

2. Results

In order to examine whether cellular processes like APP processing, degradation, oxidative stress, neuroinflammation, lipid- and energy-metabolism, G-protein mediated signaling, neurogenesis and transcriptional regulation which are impaired in AD, are affected by mild to moderate vitamin D hypovitaminosis, we decided to analyse the expression of the AD-related genes listed in table 2 in mouse brains with a mild to moderate vitamin D hypovitaminosis. For further information, a reference to the potential link to AD is given in table 2.

Table 2. Gene expression alterations in mild to moderate vitamin D hypovitaminosis: gene selection.

pathway	gene	gene name	reference
APP homeostasis	*Ace*	angiotensin I converting enzyme	[25]
	Adam9	a disintegrin and metallopeptidase domain 9	[26]
	Adam17	a disintegrin and metallopeptidase domain 17	[26]
	Apba1	amyloid beta precursor protein binding, family A, member 1	[27]
	Apba3	amyloid beta precursor protein binding, family A, member 3	[27]
	Apbb1	amyloid beta precursor protein binding, family B, member 1	[28]
	Apbb2	amyloid beta precursor protein binding, family B, member 2	[28]
	Aph1a	aph1 homolog A, gamma secretase subunit	[29]
	Aph1b	aph1 homolog B, gamma secretase subunit	[29]
	Aph1c	aph1 homolog C, gamma secretase subunit	[29]
	App	amyloid beta precursor protein	[30]
	Appbp1	amyloid beta precursor protein binding protein 1	[31]
	Bace1	beta-site APP-cleaving enzyme 1	[32]
	Bace2	beta-site APP-cleaving enzyme 2	[33]
	Cdkl1	cyclin-dependent kinase-like 1	[34]
	Ctsb	cathepsin B	[35]
	Ctsg	cathepsin G	[36]
	Ctsl	cathepsin L	[37]
	Ece1	endothelin converting enzyme 1	[38]
	Ece2	endothelin converting enzyme 2	[38]
	Hadh2	3-hydroxyacyl-CoA dehydrogenase	[39]
	Ide	insulin degrading enzyme	[40]
	Lrp1	low density lipoprotein receptor-related protein 1	[41]
	Mmp2	matrix metallopeptidase 2	[42]
	Mmp9	matrix metallopeptidase 9	[42]
	Ncstn	nicastrin	[43]
	Nep	membrane metallo endopeptidase	[40]
	Pkp4	plakophilin 4	[44]

	Plg	plasminogen	[45]
	Prnp	prion protein	[46]
	Psen1	presenilin 1	[43]
	Psen2	presenilin 2	[43]
	Psenen2	presenilin enhancer 2, gamma secretase subunit	[43]
	Psmb5	proteasome (prosome, macropain) subunit, beta type 5	[47]
	Serpina3a	Serpina3a serine (or cysteine) peptidase inhibitor, clade A, member 3A	[48]
	Snca	synuclein, alpha	[49]
	Sncb	synuclein, beta	[50]
	Ubqln1	ubiquilin 1	[51]
oxidative stress	*Als2*	amyotrophic lateral sclerosis 2	[52]
	Casp3	caspase 3	[53]
	Cat	catalase	[54]
	Cdk5	cyclin-dependent kinase 5	[55]
	Duox1	dual oxidase 1	[56]
	Epx	eosinophil peroxidase	[57]
	Ercc2	excision repair cross-complementing rodent repair deficiency, complementation group 2	[58]
	Ercc6	excision repair cross-complementing rodent repair deficiency, complementation group 6	[59]
	Gab1	GRB2-associated binding protein 1	[60]
	Gpx1, 2, 3, 5, 6, 7	glutathione peroxidase 1, 2, 3, 5, 6, 7	[61]
	Nqo1	NAD(P)H dehydrogenase, quinone 1	[62]
	Nudt15	nudix type motif 15	[63]
	Park7	parkinson disease (autosomal recessive, early onset) 7	[64]
	Ppp1r15b	protein phosphatase 1, regulatory subunit 15B	[65]
	Prdx1 / 2 / 6	peroxiredoxin 1	[66]
	Sod1	superoxide dismutase 1, soluble	[67]
	Txnip	thioredoxin interacting protein	[68]
	Txnrd2	thioredoxin reductase 2	[69]
	Ucp3	uncoupling protein 3	[70]
	Uqcrc1	ubiquinol-cytochrome c reductase core protein I	[71]
	Uqcrc2	ubiquinol-cytochrome c reductase core protein II	[72]
	Xpa	xeroderma pigmentosum, complementation group A	[73]
lipid metabolism	*Aass*	aminoadipate-semialdehyde synthase	[74]
	Abca1	ATP-binding cassette, sub-family A, member 1	[75]
	Acat1-3	acetyl-CoA acetyltransferase 1-3	[76]
	Apoa1	apolipoprotein A-I	[77]
	Apoe	apolipoprotein E	[78]

	Clu	clusterin	[79]
	Hmgcr	3-hydroxy-3-methylglutaryl-CoA reductase	[80]
	Lrp6	low density lipoprotein receptor-related protein 6	[81]
	Lpl	lipoprotein lipase	[82]
kinases/ phosphatases/ G-proteins	*Prkca*	protein kinase C, alpha	[83]
	Prkcb1 / d / e / g	protein kinase C, beta 1 / delta / epsilon / gamma	[84]
	Prkc1	serine/threonine protein kinase	[85]
	Prkcq	protein kinase C, theta	[86]
	Prkcz	protein kinase C, zeta	[87]
	Cdc2	cyclin-dependent protein kinase 2	[88]
	Gnao1	guanine nucleotide binding protein, alpha O	[89]
	Gnb1-5	guanine nucleotide binding protein (G protein), beta polypeptide 1-5	[90]
neurogenesis	*Map2*	microtubule-associated protein 2	[91]
	Mapt	microtubule-associated protein tau	[92]
	Plat	plasminogen activator, tissue	[93]
	Plau	plasminogen activator, urokinase	[94]
neurotransmission	*Ache*	acetylcholinesterase	[95]
	Bche	butyrylcholinesterase	[96]
	Chat	choline acetyltransferase	[97]
	Mpp4	membrane protein, palmitoylated 4	[98]
	Bdnf	brain-derived neurotrophic factor	[99]
energy metabolism	*Prkaa1*	protein kinase, AMP-activated, alpha 1 catalytic subunit	[100]
	Prkaa2	protein kinase, AMP-activated, alpha 2 catalytic subunit	[101]
	Idh1	isocitrate dehydrogenase 1 (NADP+), soluble	[102]
	Ins	insulin	[103]
	Ern1	endoplasmic reticulum to nucleus signaling 1	[104]
	Tpo	thyroid peroxidase	[105]
	Insr	insulin receptor	[106]
regulation of gene expression	*Ep300*	E1A (adenovirus early region 1A) binding protein p300	[107]
	Hdac1	histone deacetylase 1	[108]
neurofibrillary tangles	*Gap43*	growth associated protein 43	[109]
	Gsk3a	glycogen synthase kinase 3 alpha	[110]
	Gsk3b	glycogen synthase kinase 3 beta	[111]
inflammation	*A2m*	alpha-2-macroglobulin	[111]
	Il1A	interleukin 1, alpha	[112]
	Casp4	caspase 4	[113]
	Mpo	myeloperoxidase	[114]

Analysing the 117 selected genes (see table 2) by RT-PCR for changes in their expression level in a first mouse population, containing four wildtype (wt) and four vitamin D deficient mice, shows that expression level of 25 out of these 117 genes were changed with a significance of $p \leq 0.1$ by the vitamin D deficit and 92 genes showed an alteration with a significance of $p \geq 0.1$ (see table 3). The 25 top target candidates of the first mouse cohort were further analysed in a second mouse population containing three wt and three vitamin D deficient mice.

Table 3. Expression changes of 117 AD-related genes in mild to moderate vitamin D hypovitaminosis (n=4).

gene	% of control	standard error	*t* test	
Pkp4	77.95	1.25	0.000	
Mpp4	52.79	5.06	0.000	
Plat	63.75	4,28	0.000	
Apba1	65.91	4.59	0.000	
Ppp1r15b	69.67	5.87	0.001	
Nep	67.08	8.07	0.007	
Prkcd	65.99	8.40	0.007	
Aph1c	70.55	7.76	0.009	
Acat3	144.78	12.90	0.013	
Casp4	162.99	18.36	0.014	
Gap43	89.39	3.42	0.021	
Apbb2	74.42	8.55	0.024	
Ep300	66.96	11.21	0.026	
Gnb5	63.32	12.79	0.029	
Psmb5	161.47	21.96	0.031	
Gnb4	65.24	14.30	0.051	
Gnao1	82.36	7.41	0.055	
Snca	140.27	18.53	0.073	
Acat1	195.21	44.09	0.074	
Nqo1	80.66	9.09	0.077	
Park7	74.73	11.99	0.080	
Prkce	72.35	13.13	0.080	
Gpx5	166.18	33.29	0.094	
Abca1	157.51	29.09	0.095	
Insr	67.25	16.77	0.099	
Prkaa2	79.18	11.68	0.125	
Sncb	82.63	10.24	0.141	
Map2	93.73	4.04	0.171	

selected top candidate genes for analysis of second mouse cohort

Cdkl1	93.72	4.06	0.173
Ece2	86.08	9.02	0.174
Xpa	70.31	19.40	0.177
Lpl	119.20	12.59	0.178
Ctsg	333.69	159.88	0.194
Gpx6	139.95	28.36	0.209
Ins	210.65	78.88	0.210
Acat2	159.74	42.99	0.214
Bche	146.34	33.47	0.215
Prkca	81.63	13.29	0.216
Apbb1	87.10	9.38	0.218
Serpina3a	260.19	121.19	0.234
Clu	86.13	10.53	0.236
Mmp2	146.13	36.96	0.258
Aph1b	92.73	5.84	0.260
Cdk5	113.68	11.63	0.284
Ctsb	146.67	40.77	0.296
Epx	111.53	10.53	0.316
Hdac1	151.76	47.93	0.322
Ide	153.98	50.94	0.330
Ece1	78.59	20.34	0.333
Prdx1	84.55	15.34	0.353
Bace2	126.88	27.15	0.360
Gpx7	132.70	33.09	0.361
Ern1	115.60	15.79	0.362
Idh1	143.13	43.99	0.365
Ucp3	490.65	412.05	0.380
Gsk3b	170.56	75.52	0.386
Apba3	84.97	16.40	0.395
Plg	222.90	134.94	0.398
Adam9	89.38	11.79	0.403
Plau	78.38	24.54	0.412
Lrp6	107.60	8.78	0.420
Mpo	157.64	70.46	0.445
Chat	146.56	59.33	0.462
Txnip	135.23	48.89	0.498
Prkc1	119.69	27.66	0.503
Prkcq	127.20	39.09	0.513
Mapt	102.27	3.39	0.528
Il1a	120.84	31.28	0.530
Gpx1	86.52	21.48	0.554

Prkcg	85.51	23.42	0.559
Adam17	113.92	22.74	0.563
Bdnf	111.42	18.80	0.566
Lrp1	96.86	5.24	0.570
Cat	109.43	15.82	0.573
Psen2	112.84	22.02	0.581
Hmgcr	138.22	67.83	0.593
Prdx2	108.25	15.37	0.611
Ercc6	107.48	14.62	0.627
Appbp1	105.83	11.53	0.631
Ctsl	95.03	9.95	0.635
Prkcb1	91.20	18.67	0.654
Uqcrc2	122.45	48.07	0.657
Ncstn	115.11	32.38	0.657
Ubqln1	107.95	17.20	0.660
Ache	95.31	10.29	0.664
Tpo	129.25	65.43	0.671
Prkaa1	109.64	22.06	0.678
Uqcrc1	107.28	17.45	0.691
Apoa1	124.69	60.01	0.695
Cdc2	92.96	17.12	0.695
Apoe	93.91	15.42	0.706
Hadh2	115.69	40.94	0.715
App	112.38	32.59	0.717
Psenen2	108.82	24.80	0.734
Mmp9	93.62	18.02	0.736
Aass	107.73	22.24	0.740
Bace1	96.45	10.48	0.746
Duox1	112.77	38.29	0.750
Gab1	94.45	16.76	0.752
Gpx2	115.72	50.49	0.766
Als2	112.12	39.76	0.771
Prdx6	104.81	17.60	0.794
Ace	109.54	36.98	0.805
Txnrd2	94.19	22.84	0.808
Aph1a	103.96	15.65	0.809
Prkcz	114.45	59.68	0.817
A2m	106.50	39.10	0.874
Gnb2	106.41	41.28	0.882
Gsk3a	97.53	16.92	0.889
Psen1	92.27	54.56	0.892

Gpx3	97.02	28.02	0.919
Sod1	101.63	16.64	0.925
Gnb1	101.74	26.65	0.950
Prnp	101.35	30.06	0.966
Nudt15	101.09	30.20	0.972
Casp3	99.58	15.47	0.979
Ercc2	99.77	10.05	0.982

In table 4 the combined expression data of both mouse populations are listed.

Table 4. Combined analysis of mouse population 1 and 2 of the 25 most promising AD-related target genes selected by the results of the first mouse cohort.

gene	% of control	standard error	*t* test	
Nep	77.91	6.80	0.0069	selected genes for further analysis with additional three housekeeping genes
Acat1	274.22	63.29	0.0175	
Psmb5	164.04	24.53	0.0228	
Casp4	154.69	21.03	0.0232	
Plat	75.05	8.23	0.0290	
Snca	154.86	24.24	0.0430	
Ep300	76.46	11.19	0.0572	
Park7	80.66	9.34	0.0607	
Gnb5	78.94	10.20	0.0613	
Apba1	81.24	10.29	0.0933	
Abca1	343.84	134.99	0.0960	
Gpx5	159.41	33.65	0.1029	
Gnb4	79.26	11.97	0.1089	
Acat3	127.88	17.11	0.1293	
Apbb2	87.94	10.09	0.2551	
Gap43	116.17	18.41	0.3968	
Nqo1	125.61	29.18	0.3973	
Insr	87.18	15.57	0.4262	
Prkcd	88.15	18.16	0.5263	
Aph1c	86.41	20.90	0.5278	
Mpp4	86.28	24.49	0.5857	
Prkce	94.20	15.97	0.7226	
Ppp1r15b	108.62	30.55	0.7827	
Pkp4	96.93	12.01	0.8029	
Gnao1	96.67	14.48	0.8221	

As shown in table 4, eleven genes were affected ($p \leq 0.1$) by the moderate vitamin D deficit. Expression of *Nep*, *Plat*, *Ep300*, *Park7*, *Gnb5*, *Apba1* was reduced (*Nep*: 77.91% ± 6.80%, p = 0.007; *Plat*: 75.05% ± 8.23%, p = 0.029; *Ep300*: 76.46% ± 11.19%, p = 0.057; *Park7*: 80.66% ± 9.34%, p = 0.061; *Gnb5*: 78.94% ± 10.20%, p = 0.061; *Apba1*: 81.24% ± 10.29%, p = 0.093), whereas expression of *Acat1*, *Psmb5*, *Casp4*, *Snca*, *Abca1* was elevated in brain samples of hypovitaminosis D mice compared to control group (*Acat1*: 274.22% ± 63.29%, p = 0.018; *Psmb5*: 164.04% ± 24.53%, p = 0.023; *Casp4*: 154.69% ± 21.03%, p = 0.023; *Snca*: 154.86% ± 24.24%, p = 0.043; *Abca1*: 343.84% ± 134.99%, p = 0.096). Corresponding box plots are pictured in figure 2 (or in supplement figure 1).

The expression data illustrated in figure 2 showing the most promising candidates of AD-related genes affected by mild to moderate vitamin D hypovitaminosis were obtained by RT-PCR analysis compared to the housekeeping gene actin beta (*Actb*). To further strengthen our findings we performed RT-PCR analysis of these genes compared to three additional housekeeping genes, ATP synthase subunit beta (*Atp5b*), myosin phosphatase Rho interacting protein (*Mprip*) and RNA polymerase II subunit F (*Polr2f*) (table 5). The determination of the combined expression level compared to all four housekeeping genes – *Actb*, *Atp5b*, *Mprip* and *Polr2f* – revealed that eight out of the eleven obtained candidate genes were significantly altered. *Plat*, *Gnb5*, *Nep* and *Park7* were significantly decreased (*Plat*, mean: 70.4% ± 2.91%, $p \leq 0.001$; *Gnb5*, mean: 77.41% ± 3.44%, p = 0.0006; *Nep*, mean: 83.73% ± 4.50%, p = 0.0112; *Park7*, mean: 88.10% ± 4.16%, p = 0.0288), *Psmb5*, *Casp4*, *Snca* and *Acat1* were significantly elevated (*Psmb5*, mean: 173.90% ± 11.96%, p = 0.0008; *Casp4*, mean: 194.77% ± 16.06%, p = 0.0011; *Snca*, mean: 160.19% ± 10.43%, p = 0.0012; *Acat1*, mean: 214.06% ± 23.12%, p = 0.0026). Expression of *Abca1* was also still increased, however did not reach significance when compared to all four housekeeping genes (mean: 201.79% ± 47.96%, p = 0.0780) (table 5).

Table 5. Expression changes of the eleven selected AD-related candidate genes of the combined two mouse cohorts with mild to moderate vitamin D hypovitaminosis compared to four housekeeping genes.

gene	*Actb*	*Atp5b*	*Mprip*	*Polr2f*	mean	*t* test
Plat	*75.05% ± 8.23%*	*65.83% ± 6.52%*	*75.80% ± 9.81%*	*64.93% ± 5.34%*	*70.40% ± 2.91%*	*0.0001*
Gnb5	*78.94% ± 10.20%*	*74.73% ± 21.66%*	*86.10% ± 25.17%*	*69.86% ± 15.03%*	*77.41% ± 3.44%*	*0.0006*
Psmb5	*164.04% ± 24.53%*	*155.92% ± 30.05%*	*166.48% ± 33.06%*	*209.14% ± 80.91%*	*173.90% ± 11.96%*	*0.0008*

Casp4	*154.69% ± 21.03%*	*188.86% ± 49.24%*	*231.84% ± 88.30%*	*203.69% ± 39.23%*	*194.77% ± 16.06%*	*0.0011*
Snca	*154.86% ± 24.24%*	*139.15% ± 22.89%*	*157.74% ± 39.70%*	*188.99% ± 65.07%*	*160.19% 10.43%*	*0.0012*
Acat1	*274.22% ± 63.29%*	*173.44% ± 67.33%*	*226.12% ± 116.62%*	*182.46% ± 56.49%*	*214.06% ± 23.12%*	*0.0026*
Nep	*77.91% ± 6.80%*	*76.19% ± 7.41%*	*84.79% ± 15.14%*	*96.03% ± 23.06%*	*83.73% ± 4.50%*	*0.0112*
Park7	*80.66% ± 9.34%*	*81.14% ± 13.59%*	*95.51% ± 26.04%*	*95.10% ± 15.76%*	*88.10% ± 4.16%*	*0.0288*
Abca1	*343.84% ± 134.99%*	*135.55% ± 25.71%*	*154.96% ± 42.88%*	*172.81% ± 54.78%*	*201.79% ± 47.96%*	*0.0780*
Ep300	*76.46% ± 11.19%*	*100.21% ± 28.29%*	*126.13% ± 53.29%*	*106.03% ± 20.30%*	*102.21% ± 10.22%*	*0.8361*
Apba1	*81.24% ± 10.29%*	*98.07% ± 14.33%*	*114.23% ± 29.39%*	*111.14% ± 14.42%*	*101.17% ± 7.51%*	*0.8813*

Summary table 6 shows a list of the changed genes categorized by cellular pathways involved in AD pathogenesis.

Table 6. Gene expression changes in mild to moderate vitamin D hypovitaminosis: AD-related pathways.

pathway	Alzheimer's Disease	hypovitaminosis D
APP homeostasis	increased Aβ plaque formation [115, 116]	*Snca* ↑
	decreased Aβ degradation [117]	*Nep* ↓, *Psmb5* ↑
oxidative stress	changed stress sensor [118]	*Park7*↓
inflammation	triggered innate immune response [119, 120]	*Casp4* ↑
lipid metabolism	changed cholesterol metabolism [121, 122]	(*Abca1* ↑), *Acat1* ↑
signal transduction	decreased G-proteins [123]	*Gnb5*↓
neurogenesis	activated tPA/plasmin system [93]	*Plat* ↓

3. Discussion

Besides the two main pathological hallmarks of AD, extracellular Aβ-plaques and intracellular neurofibrillary tangles, further critical metabolic processes are affected in AD, including neuroinflammation, oxidative stress, lipid homeostasis, G-protein mediated signaling, neurogenesis and transcriptional regulation [121, 123-127]. Importantly, no causal treatment is available emphasizing the need for efficient prevention and/or cure for AD. An innovative approach for the treatment of AD is demonstrated by the therapeutic potential of lipids or liposoluble vitamins like vitamin E and D or its analogues [11, 22, 128-130]. Vitamin D hypovitaminosis, affecting up to 85% of the elderly population in the industrial nations [17], was linked to an increased risk for neurodegenerative diseases like AD [20, 21, 131]. AD patients show lower concentrations of circulating serum 25(OH) vitamin D_3 and reveal differences in *VDR* expression or polymorphisms compared to matched controls [15, 132, 133]. Furthermore, recent clinical studies reported a higher prevalence of dementia in context with vitamin D insufficiency. Interestingly, they found lower serum vitamin D levels in dark-skinned than in fairer-skinned individuals [23]. A possible explanation could be the skin pigmentation because the higher concentration of melanin decelerated the synthesis of vitamin D [134]. With simultaneous consideration of these facts, a higher prevalence of dementia could be expected in dark-skinned individuals. And indeed a recent study with 2.5 million participants demonstrated a 28 % higher incidence of dementia diagnosis in men of African descent compared to those of Caucasian descent [135]. Besides the skin color, the geographical variation also seems to influence the risk of dementia. Recent findings showed higher rates of dementia in the north of the northern hemisphere relative to the south, discussed to be due to a reduced sunlight exposure resulting in decreased vitamin D levels [136].

As vitamin D is able to influence the expression of target genes via the VDR, the aim of our study was to examine if the expression of AD-related genes is changed under mild to moderate hypovitaminosis conditions. We analysed the expression of 117 genes, which play a role in APP homeostasis, oxidative stress, inflammation, lipid metabolism, G-proteins, neurogenesis or transcriptional regulation, in brains of mice with a mild to moderate vitamin D hypovitaminosis (see table 2). Gene expression of eight genes, summarized and attributed to their pathway in table 6, were significantly altered in vitamin D deficient mouse brains when normalized to four housekeeping genes, to exclude potential false positive AD-related target genes affected by mild to moderate vitamin D hypovitaminosis by using only one housekeeping gene. *Plat*, *Gnb5*, *Psmb5*, *Casp4*, *Snca*, *Acat1*, *Nep* and *Park7* still revealed highly significant alterations. In our recent study a 20% to 30% vitamin D reduction in mouse brain was utilized to examine the effect of a mild to moderate vitamin D hypovitaminosis in respect to AD.

In the following paragraphs the pathways affected by vitamin D hypovitaminosis and their role in AD are briefly discussed.

APP homeostasis

It is well known, that APP homeostasis is impaired towards increased Aβ plaque formation and decreased Aβ degradation in AD [115, 117]. Besides several other factors, Aβ levels are increased in AD due to an upregulated expression of Aβ generating enzymes and a downregulated expression of Aβ degrading enzymes [137, 138]. Analysing the effect of vitamin D deficiency on Aβ degradation, we found a significantly reduced *Nep* expression as well as a significantly upregulated expression of *Psmb5* (see table 5). The zinc metalloendopeptidase neprilysin is one of the most prominent Aβ degrading enzymes [139]. The decreased expression of *Nep* as a consequence of the vitamin D deficit (*Nep*: 83.73% ± 4.50%, p = 0.0112) is in accordance to a previous study revealing a 17% reduction of *Nep* expression in hypovitaminosis D mouse brains compared to control mouse brains [22].

It has to be emphasized that besides the other unknown newly identified targets, *Nep* and *Park7* have already been reported to be affected be vitamin D. We decided to include these two known genes in our study to evaluate whether a 20-30% vitamin D hypovitaminosis is sufficient to reproduce the known alterations in gene expression. Moreover, in our study the gene expression was analysed in mouse brain. Up until now, very little was known about vitamin D also playing a role in gene expression in brain. By identifying *Nep* and *Park7* as targets of vitamin D hypovitaminosis we could, in line with the previous studies, also demonstrate that in principal vitamin D hypovitaminosis is able to modulate gene expression in brain as well and not only in liver or other organs. A strong reduction in *Nep* mRNA levels has also been reported in microglia from 14 months old PS1-APP transgenic mice [140]. Notably, in a recent meta-analysis the level of *NEP* mRNA is described to be significantly lower in AD cases than in non-AD cases [141].

In association with Aβ plaques an accumulation of aberrant and ubiquitinated proteins is found in AD [142]. Besides Aβ degradation involving NEP, the ubiquitin proteasomal system (UPS) degrades abnormal or misfolded proteins. Changes in this cellular system lead to the accumulation of proteins and to neuronal disorders like AD [47, 143]. Under mild to moderate hypovitaminosis D, the expression of *Psmb5*, encoding for the proteolytic constitutive proteasome subunit beta 5 and exhibiting chymotrypsin-like activity, was significantly increased to 173.90%. Elevated chymotryptic and tryptic (encoded by the b-subunit b2) proteasome activity has also been reported for astrocytes treated with Aβ42 for 24 hours [144], indicating that proteasome activity is elevated in AD and an impaired proteasome function has been reported for *post mortem* brains of AD patients [145, 146] and for several AD mouse models [147-149]. Studies addressing the expression of the constitutive proteasome subunits in AD reported an overall decrease of β5 (*PSMB5*) expression at all Braak stages [150].

Furthermore, we found that the expression of *Snca*, encoding the alpha-synuclein protein was significantly upregulated in brains of mice with a mild to moderate vitamin D hypovitaminosis (*Snca*: 160.19% ± 10.43%, p = 0.0012). Interestingly, SNCA is discussed to interact with Aβ and to stimulate aggregation of Aβ [151-155]. A recent study reports an elevated mRNA expression of *SNCA* in peripheral leukocytes of AD patients compared to age- and gender-matched control individuals [49]. Besides the impact of SNCA in AD, a tight association of the neuropathological hallmarks of PD and Lewy body disease with SNCA has been shown [156]. In summary we could demonstrate that the altered expression of *Nep*, *Psmb5* and *Snca* indicates that APP homeostasis is impaired by vitamin D hypovitaminosis. An impaired APP homeostasis is known to be a key process in AD highlighting a potential causal link between AD and hypovitaminosis D. Nevertheless this potential mechanistic link and the link discussed below has to be proven in clinical studies dealing with vitamin D supplementation.

Oxidative stress

In addition to elevated Aβ levels resulting in senile plaques, increased oxidative stress [157, 158] is a major feature contributing to AD pathology. In our study we observed changed expression of *Park7* in brains of vitamin D deficient mice (*Park7*: 88.10% ± 4.16%, p = 0.0288). The cancer- and Parkinson´s disease (PD) associated protein PARK7 acts as oxidative stress sensor and is involved in neuroprotective mechanisms [64, 159]. A study, analyzing the *Park7* expression in human *post mortem* AD brains, reported an increased protein expression [160]. In respect to oxidative stress, it was shown that vitamin D can prevent Aβ-induced inducible nitric oxide synthase (iNOS) expression via VDR [161].

Inflammation

An activation of inflammatory pathways is clearly linked to AD pathogenesis [10, 119, 162]. Expression of *Casp4*, encoding caspase 4, a member of the cysteine-aspartic acid protease family, was significantly increased in mild to moderate hypovitaminosis D (*Casp4*: 194.77% ± 16.06%, p = 0.0011). Caspase 4 is described to be involved in Aβ-induced cell death [163] and an upregulated *Casp4* expression was reported in an AD mouse model [113]. Interestingly, it could be shown, that calcitriol, the double-hydroxylated active form of vitamin D_3, rebalances inflammation to promote Aβ phagocytosis *in vitro* [164]. This data indicates that CASP4 activity is affected both in AD and hypovitaminosis D.

Lipid metabolism

Besides the already discussed metabolic processes affected in AD, more and more evidence arises, that AD pathology is closely linked to changes in lipid metabolism. APP and its processing enzymes are all transmembrane proteins [121] and several lipid classes affect Aβ generation [1, 128, 165-167] or have been found to be altered

in AD *post mortem* brains [5, 168-174]. We observed two genes affected by vitamin D deficit, *Abca1* and *Acat1*. Interestingly, *Acat1* and *Abca1* both involved in cholesterol homeostasis showed the highest effect strength, however *Abca1* slightly failed to reach significance. *Abca1* encodes a protein called ATP binding cassette subfamily A member 1, which plays a crucial role in cellular trafficking of cholesterol [175]. We observed an increased expression of *Abca1* in vitamin D hypovitaminosis (*Abca1*: 201.79% ± 47.96%, p = 0.0780), however statistical analysis did not reach significance. *ABCA1* expression has also been found to be elevated in AD hippocampal neurons [176].

The second lipid metabolism related gene, which was affected by hypovitaminosis D, was *Acat1*, which encodes the acetyl-CoA acetyltransferase 1. Acetoacetyl-CoA can be metabolized by HMG-CoA synthase resulting in 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) which in turn can be further metabolized to cholesterol, which has been closely linked to AD pathology [128]. Elevated cholesterol level have been found to increase Aβ production whereas decreased levels strongly reduce Aβ release *in vitro* and *in vivo* [177-180]. Our data showed a significant elevated *Acat1* expression in mild to moderate hypovitaminosis D (*Acat1*: 214.06% ± 23.12%, p = 0.0026) which could result in increased cholesterol and finally Aβ level. A potential explanation for the increased *Abca1* expression in hypovitaminosis D might be the altered cholesterol homeostasis caused by an increased *Acat1* expression, because it has been reported that cholesterol upregulates *Abca1* gene expression [175]. In summary vitamin D hypovitaminosis affects gene expression of lipid genes, especially in cholesterol homeostasis providing a general potential mechanistic link to neurodegenerative diseases especially AD.

Signal transduction

Guanine nucleotide-binding proteins (G-proteins), heterotrimeric proteins composed of Gα, Gβ and Gγ subunits, function in signal transduction pathways and are cellular switches because of their ability to bind to and hydrolyze guanosine triphosphate to guanosine diphosphate [181]. Our study showed a significantly reduced expression of *Gnb5* in mild to moderate hypovitaminosis D (*Gnb5*: 77.41% ± 3.44%, p = 0.0006). *Gnb5* encodes for the G-protein subunit β5, which forms a heterodimer with the γ-subunit and regulates the α-subunit. A serial analysis of gene expression (SAGE) study in human hippocampus reported a downregulated expression of the G-protein signaling molecule Gnb5 in APOE3/4 and APOE4/4 AD patients [89]. Taking into consideration that G-protein mediated signaling pathways including G-protein-coupled receptors (GPCRs) become important targets for potential drug treatments in neurodegenerative disorders like AD [182, 183], based on our findings vitamin D supplementation might also act via similar potentially beneficial mechanisms.

Neurogenesis

The cognitive impairments linked with neurodegeneration are one clinical hallmark of AD. Impaired neurogenesis can therefore be relevant for the progression of AD [184]. In our study on mouse brains with mild to moderate vitamin D deficit, the expression of *Plat* was significantly decreased compared to control mouse brains (*Plat*: 70.40% ± 2.91%, p = 0.0001). This gene encodes tissue-type plasminogen activator (tPA), which converts plasminogen to plasmin, and plays a role in cell migration and tissue remodeling during development and regeneration [185]. Studies using Aβ-treated primary cortical neuronal cultures showed that aggregated Aβ increased the *tPA* mRNA levels [93], whereas non-aggregated Aβ showed no effect on *tPA* expression. Notably, in this study it is further shown that plasmin degrades Aβ and inhibits Aβ neurotoxicity [93]. Decreased expression of the tPA protein in mild to moderate hypovitaminosis D would therefore decrease the generation of plasmin, finally resulting in elevated Aβ level caused by impaired Aβ degradation mediated by plasmin. Furthermore, reduced plasminogen activator-catalyzed proteolysis in neuronal tissues would influence neuronal plasticity and synaptic reorganization [185], which might contribute to the cognitive impairments found for vitamin D deficient patients [20, 186-189].

In summary it has been discussed that vitamin D also affects brain metabolism via transcriptional regulation caused by vitamin D receptor (VDR) and retinoid X receptor mediated mechanism. Although there is a clear link between AD and vitamin D hypovitaminosis, little is known whether there is an overlap of genes regulated by vitamin D homeostasis and genes affected in AD especially under mild to moderate vitamin deficiency. As 85% of the elderly western population show a vitamin D undersupply, we have decided to analyse 20% to 30% vitamin D deficient mouse brains to address the mild to moderate hypovitaminosis in humans. After verification of the top candidate genes in a second mouse population, including three additional housekeeping genes, we found eight to be significantly altered in the brain. The identified eight AD-related genes are promising target genes affected by vitamin D deficiency and are mainly involved in different metabolic pathways which all have a synergistic impact in AD (see figure 3). Further studies have to evaluate if a supplementation of vitamin D would rescue the identified impairments in gene expression. Interestingly, these pathways are not only involved in AD but also other neurodegenerative disorders like Parkinson’s disease, vascular dementia, frontotemporal disease and Lewy body disease, potentially explaining a link to these neurodegenerative diseases as well. In general our results emphasize that vitamin D homeostasis is tightly linked to several metabolic pathways not only in liver or kidney but is also abundant for a physiological brain function, an aspect which should be investigated in detail in the future.

Declaration of interest: none

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Figure caption

Figure 1. Schematic overview of the study.

Figure 2. Box plots resulting from expression analysis of *Abca1, Acat1, Apba1, Casp4, Ep300, Gnb5, Nep, Park7, Plat, Psmb5* and *Snca* in hypovitaminosis D mouse brain samples.

Figure 3. Overlap of genes and pathways affected by both, AD and mild to moderate vitamin D hypovitaminosis. All genes were significantly altered ($p \leq 0.05$) beside *Abca1* which has slightly failed to reach a significant level ($p = 0.078$).

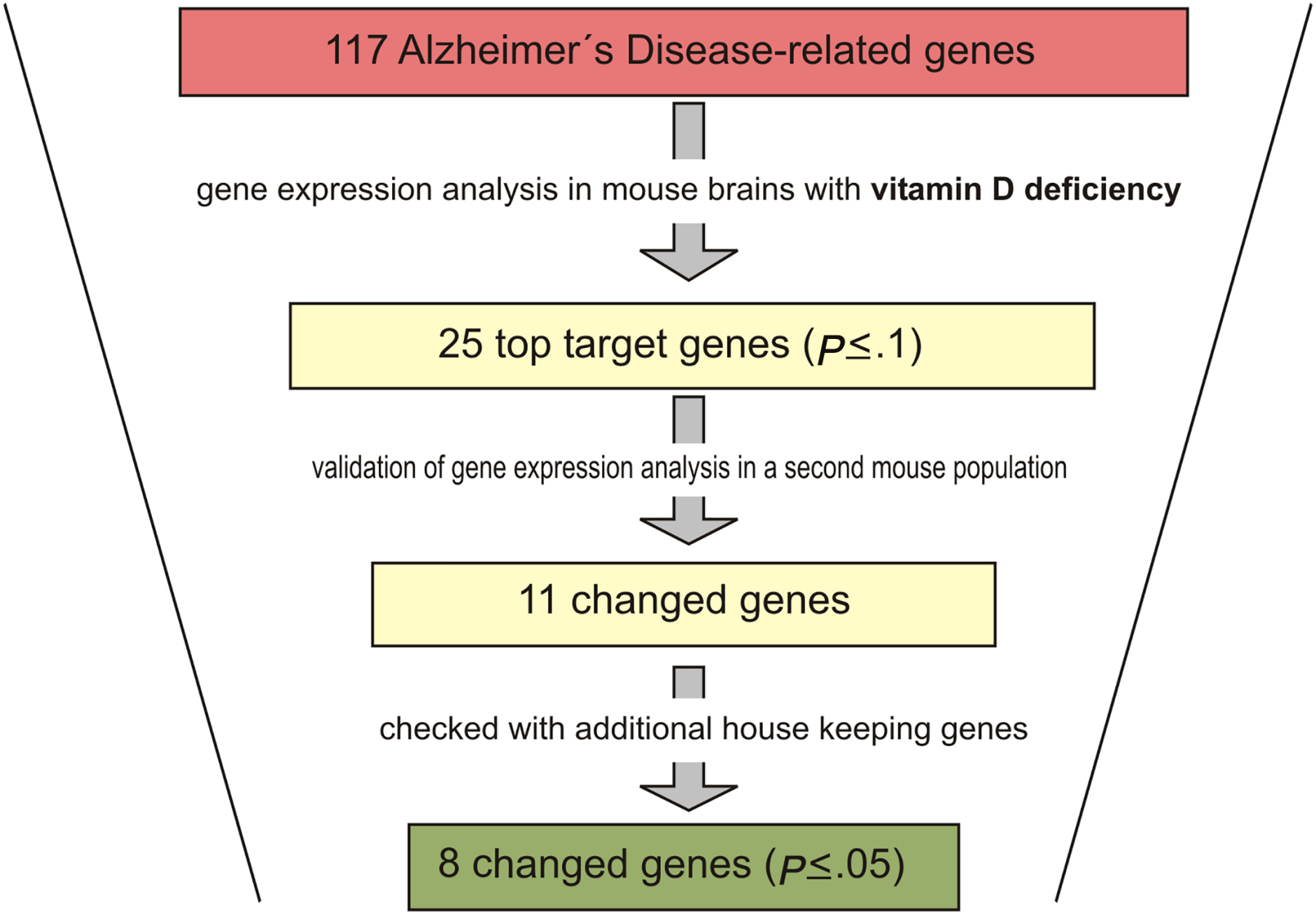
117 Alzheimer´s Disease-related genes
gene expression analysis in mouse brains with **vitamin D deficiency**
25 top target genes (*P*≤.1)
validation of gene expression analysis in a second mouse population
11 changed genes
checked with additional house keeping genes
8 changed genes (*P*≤.05)

Figure 1

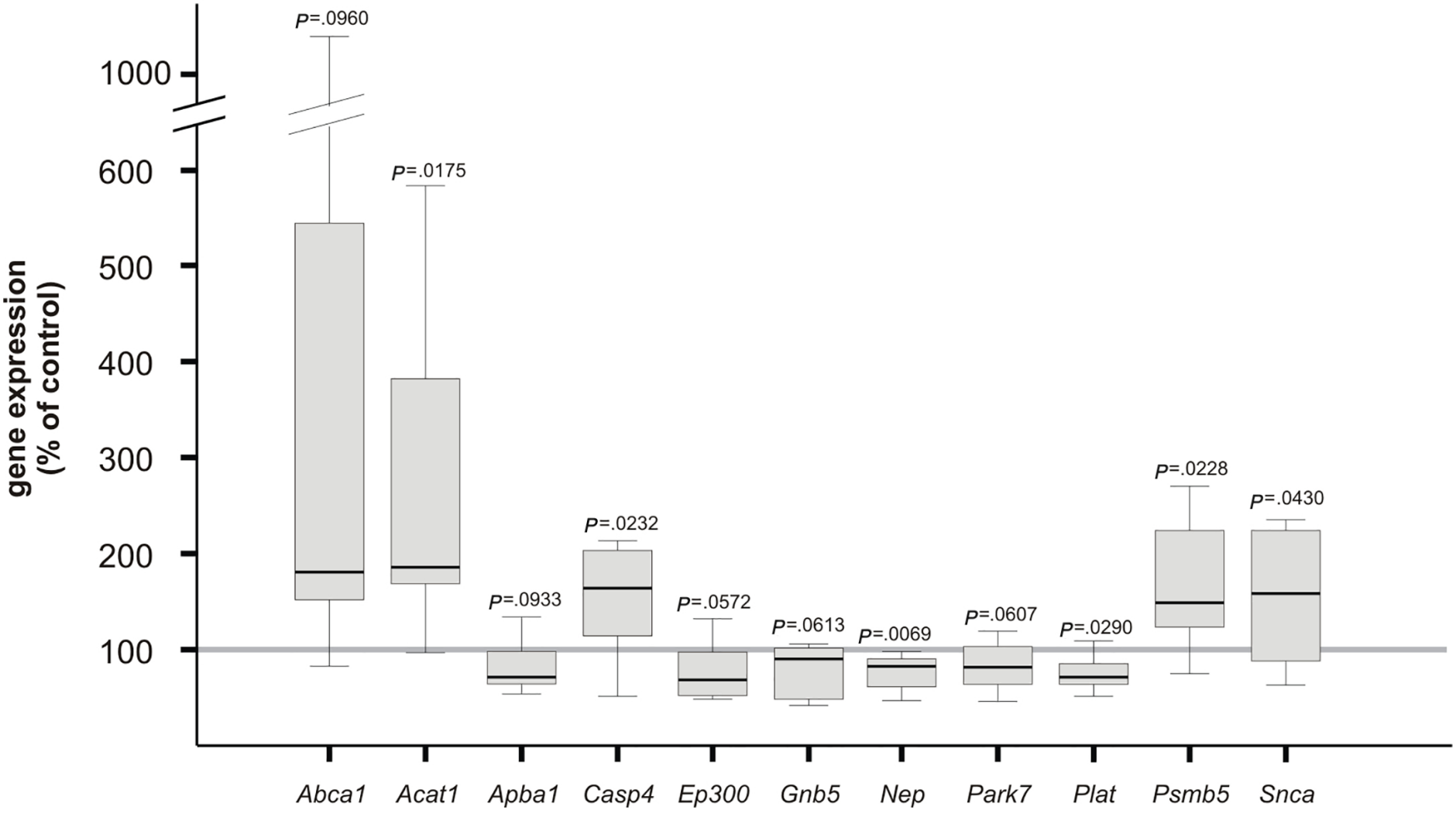
gene expression
(% of control)
1000
600
500
400
300
200
100
P=.0960
P=.0175
P=.0933
P=.0232
P=.0572
P=.0613
P=.0069
P=.0607
P=.0290
P=.0228
P=.0430
Abca1
Acat1
Apba1
Casp4
Ep300
Gnb5
Nep
Park7
Plat
Psmb5
Snca

Figure 2

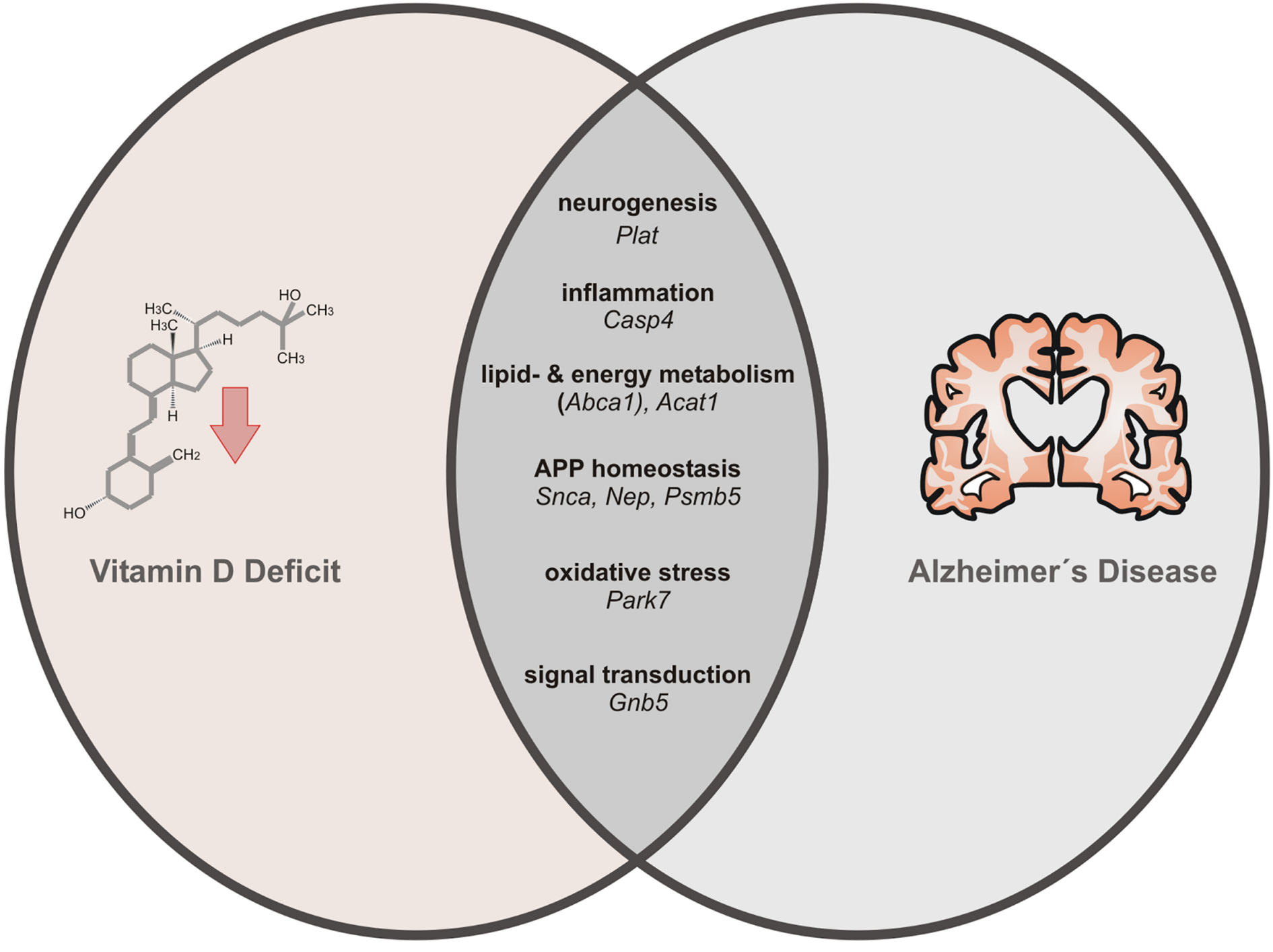
neurogenesis
Plat
inflammation
Casp4
lipid- & energy metabolism
(Abca1), Acat1
APP homeostasis
Snca, Nep, Psmb5
oxidative stress
Park7
signal transduction
Gnb5
Vitamin D Deficit
Alzheimer´s Disease

Figure 3

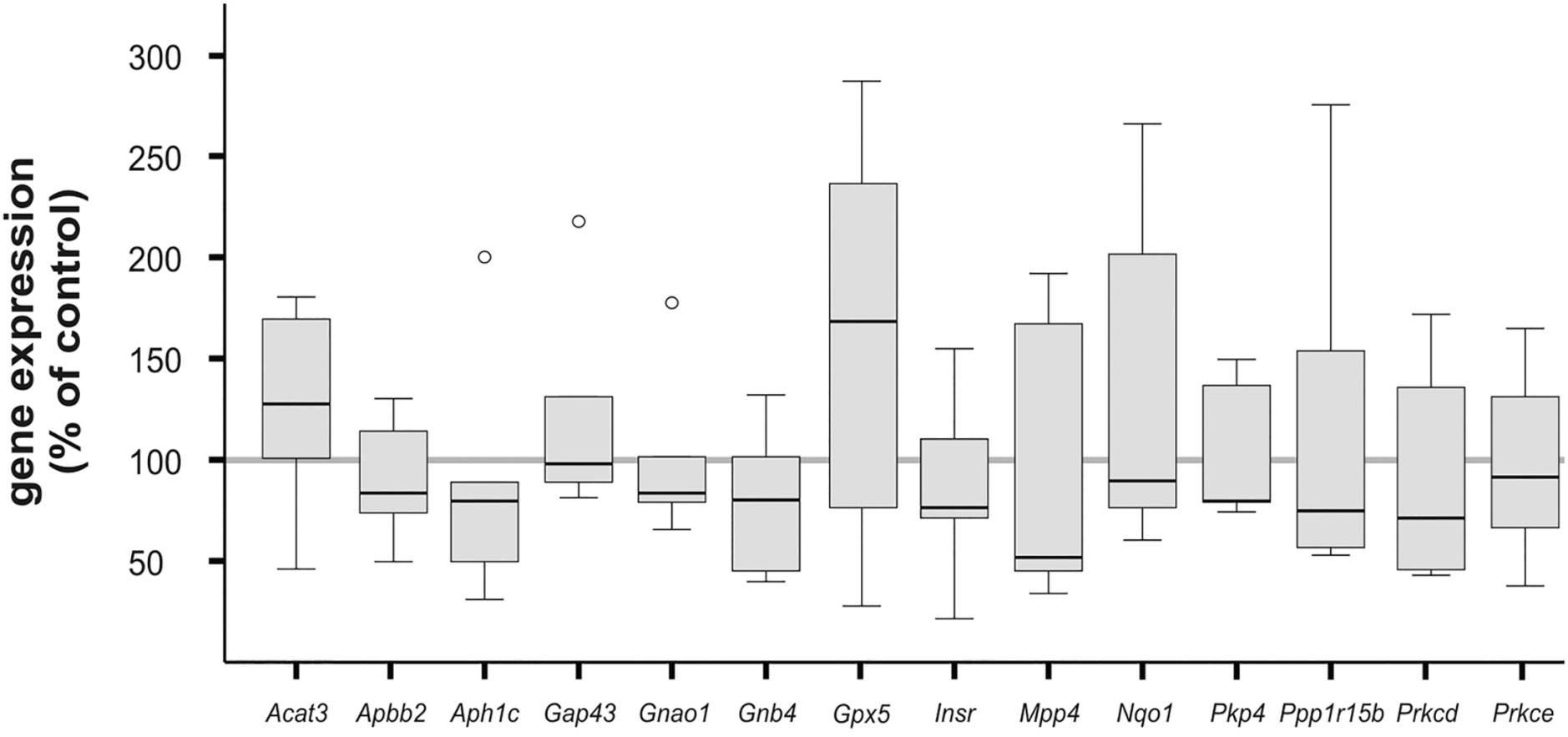
gene expression
(% of control)
300
250
200
150
100
50
Acat3
Apbb2
Aph1c
Gap43
Gnao1
Gnb4
Gpx5
Insr
Mpp4
Nqo1
Pkp4
Ppp1r15b
Prkcd
Prkce

Figure 4