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Chapter 6

Vitamin D and Sphingolipids: Role in Bone and Neural System

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Abstract

1-Alpha,25-dihydroxyvitamin D3 (1,25$(OH)_2D_3$) is known to play an important physiological role on growth and differentiation in a variety of nonmalignant and malignant cell types through classical actions, mediated by its specific receptor (VDR), and nongenomic actions resulting in the activation of specific signalling pathways. Due to the broad distribution of Vitamin D Receptor (VDR) in many tissues and the ability of 1,25$(OH)_2D_3$ to regulate fundamental processes, such as cell proliferation and differentiation, this steroid hormone has been suggested in the treatment of different diseases, from cancer to neurodegenerative diseases. In fact, structural 1,25$(OH)_2D_3$ analogues, with weaker collateral effects, have recently entered in clinical trials. Other interesting molecules due to their pleiotropic actions are the bioactive sphingolipids (SLs), in particular ceramide (Cer) and sphingosine 1-phosphate (S1P). Cells maintain a dynamic balance of these metabolites since Cer and sphingoid bases mediate cell death, while S1P exerts mitogenic effects and promotes differentiation of several cell types including osteogenic and neural cells. The biological actions of 1,25$(OH)_2D_3$ and SLs, in particular S1P, share many common effectors, including calcium regulation, growth factor expression, inflammatory cytokines, etc., but whether they could act synergistically is still unknown and deserves further investigation.

Keywords: vitamin D, sphingosine 1-phosphate, ceramide, neurodegeneration, bone

1. Introduction

1-Alpha,25-dihydroxyvitamin D3 (1,25$(OH)_2D_3$), a known regulator of calcium and phosphorus homeostasis, has also important physiological effects on growth and differentiation in a variety of nonmalignant and malignant cell types [1–4] and a central role in host defense

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against infections [5]. The classical actions of $1,25(OH)_2D_3$ start with the hormone binding to the $1,25(OH)_2D_3$ receptor/retinoic X receptor (VDR/RXR) heterodimeric complex to specific DNA sequences, whereas the rapid nongenomic actions result in the activation of specific signal transduction pathways [6, 7]. The broad distribution of VDR in the human body and the ability of $1,25(OH)_2D_3$ to control cell growth and differentiation make this hormone a potentially useful agent in the treatment of diseases, including cancer and neurodegenerative diseases. However, the systemic application of $1,25(OH)_2D_3$ is limited because of its hypercalcemic side effects [8]. Therefore, $1,25(OH)_2D_3$ analogues, with potent cell regulatory effects, but with weaker effects on calcium metabolism, have recently been obtained and some of them have entered in clinical trials [9, 10]. Sphingolipids (SLs) constitute a biologically active lipid class that is significantly important from both structural and regulatory aspects [11–14]. Indeed, they regulate fundamental cellular processes that are important in determining cellular fate, such as proliferation, apoptosis senescence, and inflammation [15–19]. Cells maintain a dynamic balance of distinct SL metabolites [20, 21], with ceramide (Cer) and sphingoid bases acting in opposite manner with respect to sphingosine 1-phosphate (S1P) that exerts mitogenic effects and promote differentiation of several cell types including skeletal muscle cells [22, 23] and neural cells [24–27]. The manipulation of SL metabolism is currently being studied as a novel strategy to regulate cell proliferation/inflammation [18, 19, 28, 29]. Interestingly, S1P can be released from many cell types including neuronal cells and osteoblasts, thus, acting as ligand of specific S1P receptors, triggers paracrine and autocrine signalling [13, 19, 30]. In the present chapter, we review the potential contribution of the biological effects of sphingolipids, $1,25(OH)_2D_3$ and its structural analogues in bone and neural disorders.

2. Vitamin D in bone physiology, osteogenesis, and osteoporosis

2.1. Bone remodelling

Bone remodelling consists in the balance between the elimination of bone due to osteoclast death and the formation of new bone by osteoblast proliferation [31]. The migration of mesenchimal stem cells (MSCs) to areas of new bone formation is also a fundamental process for skeleton maintenance. In fact, in these functional locations, MSCs, under the influence of bone morphogenetic protein, differentiate into osteoblasts. The majority of osteoblasts become osteocytes, the fully differentiated cells, within the bone matrix, where they also help in tissue repair [32]. Osteoclasts are multinucleated cells, abundant in mitochondria, vacuoles, and lysosomes [33, 34]. These cells derive from the fusion of preosteoclasts through a mechanism that is regulated by the dendritic cell-specific transmembrane protein (DC-STAMP) and the osteoclast-stimulatory transmembrane protein (OC-STAMP) [35, 36]. Osteoclasts are characterized by a ruffled border in contact with the bone surface, where the vacuolar H^+-ATPase, responsible of the maintenance of acid pH that favors the dissolution of the bone minerals, is localized. The zone beneath the ruffled borders is called resorption lacunae and is isolated from the surrounding by the sealing zone of osteoclasts that attaches the cells to the bone surface. Cathepsin K, MMP9, and tartrate resistant-acid phosphatase (TRAP), the main enzymes responsible for the degradation of bone matrix, are released in the resorption

lacunae. Matrix degradation products are endocytosed from the central portion of the ruffled border, packaged into transcytotic vesicles and secreted from the functional secretory domain [37]. The bone matrix represents an important storage of factors secreted by the osteoblasts during bone formation. Among them are the transforming growth factor β (TGFβ), the bone morphogenetic proteins (BMPs), fibroblast growth factors (FGFs), etc. They are stored in the bone matrix and serve as osteoblast-promoting components once liberated and activated by the osteoclasts.

In 1981, Rodan and Martin [38] observed that osteoblasts, but not osteoclasts, express the receptors of bone-resorbing factors, such as Parathyroid hormone (PTH) and prostaglandin E2, and proposed that osteoblasts may interfere in the process of osteoclastic resorption. Experimental evidence of the role of the microenvironment provided by osteoblastics for cell differentiation of splenic precursors, confirms this hypothesis [39]. In response to bone-resorption stimulating factors, osteoblasts produce Receptor Activator of Nuclear Factor kappa-B Ligand (RANKL), a membrane associate factor that, by binding to its receptor constitutively expressed on the surface of osteoclast precursors, stimulates cell differentiation and the activation of bone resorption. Another potential physiological regulator of bone mass is the prolin/arginine-rich end leucine-rich repeat protein (PRELP), a heparin/heparan sulfate-binding protein expressed in developing bone, cartilage, and basement membranes. PRELP inhibits osteoclast formation with a mechanism that affects the RANKL-dependent late stage of osteoclastogenesis, and its administration is reported to reduce bone loss in ovariectomized and tumor-bearing mice [40].

2.2. Effect of vitamin D on calcium homeostasis and bone remodeling

$1,25(OH)_2D_3$ was historically discovered as an anti-rachitic agent due to its effects on the demineralization process carried out by osteoclasts and intestinal calcium absorption [41]. The hormonally active form is the dihydroxylated metabolite 1,25-dihydroxyvitamin D3, or $1,25(OH)_2D_3$. It is generated by two enzymatic hydroxylation reactions, which occur first in the liver to produce 25-hydroxyvitamin D3 and second in the kidney, where both $1,25(OH)_2D_3$ and its sister metabolite 24,25-dihydroxyvitamin D3 ($24,25[OH]_2D_3$) are produced. Thus, most tissues have the ability to convert $1,25(OH)_2D_3$ into its active form, which, in turn, will bind to hormone nuclear receptor (VDR) [42]. The presence of the VDR in many tissues that are not involved in mineral metabolism indicates a wider physiological role for $1,25(OH)_2D_3$ able to positively or negatively influence target gene expression via binding of the hormone/VDR complex to specific receptor response elements, i.e., RANKL gene [43]. $1,25(OH)_2D_3$ ability to maintain serum calcium homeostasis is due to VDR-mediated signaling affecting bone physiology and intestinal calcium absorption.

In the intestine, $1,25(OH)_2D_3$-mediated processes can occur by two different mechanisms: (a) the *paracellular pathway* predominates when dietary levels of calcium are high, and it is a passive, nonsaturable diffusion process [44], calcium is transported through tight junctions, and involves several claudins proteins upregulated by $1,25(OH)_2D$ [45]; (b) the *transcellular pathway* occurs in the jejunum and duodenum in circumstances of low calcium dietary intake. It requires an active transport of calcium through the thickness of the enterocytes, and it is regulated by the subsequent involvement of transient receptor potential vanilloid

6 (TRPV6), calcium-binding protein calbindin D_{9k} (CaBP-9K), and a calcium ATPase. The transcription of these proteins is stimulated by the $1,25(OH)_2D$ signal [46]. When intestinal calcium absorption decreases, the transcellular transport in the kidney, a process similar to the active transport through the enterocytes, increases calcium reabsorption. This pathway is also stimulated by $1,25(OH)_2D_3$ [44].

In addition, in hypocalcemia conditions, parathyroid glands release PTH which, in the kidney, stimulate the production of the active form of $1,25(OH)_2D_3$. Whenever intestinal and renal calcium fluxes are insufficient to maintain the correct calcium levels in the serum, the bone represents an additional pool of calcium and the bone-resorbing process permits to adjust serum calcium concentration.

$1,25(OH)_2D_3$ activates multiple signaling pathways in bone precursor cells [42]—*nongenomic pathways* that involve: (a) the activation of voltage-sensitive calcium channels located at plasma membrane; (b) the release of calcium from intracellular stores; (c) a shift in the charge state of the matrix protein osteopontin (OPN)—*a classical nuclear receptor-mediated event* that leads to the upregulation of the OPN gene at 48 h after hormone addition. Nanomolar concentration of $1,25(OH)_2D_3$ is necessary for the nonclassical effects, while physiological $1,25(OH)_2D_3$ serum concentrations are necessary in the picomolar range [42].

Calcium balance and cell differentiation stage affect VDR action in osteogenic cells. In fact during a positive calcium balance, $1,25(OH)_2D_3$ signaling acts in a different way in osteoblasts at diverse differentiation stage: in immature osteoblasts, the hormone causes an increase in RANKL expression leading to a catabolic function on bone mass [47], whereas in mature osteoblasts, VDR stimulation produces a decrease in RANKL expression and an increase in the production of the osteoclastogenic inhibitor osteoprotegerin (OPG), which inhibits RANKL-RANK interaction [48].

During a negative calcium balance, the high RANKL/OPG ratio and the increased levels of mineralization inhibitors, such as OPN, are the two events that permit the mobilization of calcium from the bone to the serum in response to $1,25(OH)_2D_3$ and PTH signaling. By increasing calcium absorption, hormone signaling indirectly preserves bone mass mineralization. In osteocytes, VDR signaling upregulates the transcription of the bone-derived fibroblast growth factor-23 (FGF23) that stimulates TRPV5 expression, thus, increasing renal phosphate excretion [49]. Notably, FGF23 also decreases renal CYP27B1 activity, the enzyme that catalyzes the hydroxylation of $25(OH)_2D_3$ to $1,25(OH)_2D_3$ [42], avoiding overstimulation of the hormone pathway.

Osteoporosis is a systemic skeletal disease characterized by a reduction of bone mass and microarchitectural deterioration that leads to increase in bone fragility and susceptibility to fracture [50]. Osteoporosis results from the imbalance between bone resorption and bone formation, and $1,25(OH)_2D_3$ can regulate both aspects of bone turnover. Nearly in all studies, the treatment with $1,25(OH)_2D_3$ or its precursor, $1\alpha(OH)D_3$, is found to increase bone mineral density [51, 52]. More recently, the combination of $1\alpha(OH)D_3$ with an anti-resorptive *bis*-phosphonate (alendronate) enhanced bone mass with fewer falls and fractures [53].

2.3. Vitamin D deficiency and supraphysiological dose

The lack of $1,25(OH)_2D_3$ has obvious implications for human diseases. Severe $1,25(OH)_2D_3$ deficiency causes nutritional rickets in children [54] that can easily be prevented and cured by $1,25(OH)_2D_3$ supplementation [55]. Moreover, deficiency of $1,25(OH)_2D_3$ or reduced level of VDR can lead to osteomalacia disease and the recovery of a normal hormone level can resolve the disease [56]. Less severe $1,25(OH)_2D_3$ deficiency produces an increased bone turnover and an accelerated bone loss and it is associated with osteoporosis. A correlation between the sun exposure and bone physiology in regions insufficiently exposed to sunlight may involve $1,25(OH)_2D_3$ deficiency [57]. However, since $1,25(OH)_2D_3$ insufficiency is widespread, it is difficult to provide evidence regarding the direct role of sunlight on osteogenesis and osteoporosis without taking into account many other variables, such as personal bone physiology, age, weight, clothing habits, medication, and others.

Supraphysiological doses of the $1,25(OH)_2D_3$ induce calcemic side effects. In order to preserve or augment the beneficial effects of the hormone and to minimize its collateral consequences, structural analogues of $1,25(OH)_2D_3$ have been synthesized by introducing chemical modifications in the A-ring, central CD-ring region, or side chain of the hormone [58].

Some of these analogues have tissue-specific actions, exert prodifferentiating and antiproliferative effects on keratinocytes, and also possess important anti-inflammatory properties. The recently approved eldecalcitol (1a,25[OH]$_2$-2b-(3-hydroxypropyloxy)vitamin D3; ED-71; Edirol®) is an orally administered analogue of calcitriol that binds to VDR [59]. Edirol® is available for the treatment of osteoporosis [60]. The effects of this compound on bone metabolism have been reported in a randomized, open-label study in postmenopausal women, in which reductions in the markers of bone reabsorption were observed [61]. Similarly, in a randomized, noncomparative study of patients with osteoporosis, the structural analogue suppresses the biochemical markers of bone turnover in a dose-dependent manner.

Another $1,25(OH)_2D_3$ analogue with some effects on bone is ZK191784, a compound characterized by 22,23-double bond, 24R-hydroxy group, 25-cyclopropyl ring, and 5-butyloxazole-group [10]. It has been shown to exert therapeutic potential in T cell-mediated immune disorders and to significantly counteract acute and chronic intestinal inflammation [62]. While the $1,25(OH)_2D_3$ agonist effect of ZK191784 in kidney and antagonistic effect in intestine were clear, its effect on bone is still Ongoing: preliminay analyses appeared to suggest a tendency toward restoration of the reduced bone thickness in mice lacking the renal epithelial calcium channel TRPV ($Trpv5^{-/-}$) [37].

Another $1,25(OH)_2D_3$ analogue, Seocalcitol, is able to reduce the number and growth of metastasis originating from various types of cancer cells, such as bone metastasis originating from intracardially injected breast cancer cells [63]. Since $1,25(OH)_2D_3$ and its analogues possess cytostatic properties, many in vivo studies have focused on hormone analog cancer treatment combined with radiotherapy and/or chemotherapy. However, while the combination of Seocalcitol, with radiotherapy in a xenograft model for breast cancer, lead to more effective anti-cancer effects [64, 65], the combination of the analogues with chemotherapy does not always result in additive or synergistic effects. In view of the promising results that certain

hormone analogs show against cancer in vitro and in vivo animal models, some of them have been tested in cancer patients, such as Seocalcitol, which however has given rather insufficient results in clinical trials.

2.4. Animal model for bone remodeling study

Several animal models have been developed and used to understand the pathogenesis of osteoporosis and osteogenesis for the preclinical testing of new treatment options [66]. Only few of them were used in combination with $1,25(OH)_2D_3$. treatment.

The senescence accelerated mouse (SAM/P6) is a mouse model for severe osteoporosis that has low level of bone mass and develops fractures in old age [67]. It is a unique model for the study of age-related osteopenia and severe osteoporosis mimicking many aspects of the age-related changes seen in bones of humans and offering the opportunity to study relevant genes that contribute to this process.

Other studies have been performed in ovariectomy (OVX) on rats for either osteoporotic induction or fracture healing. Notably, an additive effect on bone loss was reported by combined OVX-deficient calcium or OVX-deficient $1,25(OH)_2D_3$. In addition, the rat model subjected to ovariectomy and multideficiency diet (depletion of $1,25(OH)_2D_3$, calcium, vitamin K, and phosphorus), and, thus, characterized by increased bone turnover could contribute to the study of bone- and energy metabolism in early and late stages of osteoporosis. Several ovariectomized large animals might be also used as models of osteoporosis, such as the dog, the pig, the sheep, and the nonhuman primates [68]. In particular, the sheep is also well established as a model for human bone loss/osteoporosis in orthopedic research [69]. Some limits can be the largeness in size and the difficulty to manage relatively expensive experiments.

Mouse can be a reliable animal model of glucocorticoid-induced osteopenia/osteoporosis and mimic the changes seen in humans [70]. Mice receiving glucocorticoid for a week showed an early increase in bone resorption, decreased bone mineral density, and bone mass.

Transgenic mice showing bone alterations have been also developed. Klotho mouse is a transgenic mouse model obtained by an insertion mutation that disrupts the Klotho gene locus. Klotho is a gene encoding a transmembrane protein that forms a complex with multiple fibroblast growth factor receptors and functions as coreceptor for FGF23, an osteocyte-derived hormone that induces negative phosphate balance. Defects in either Klotho or FGF23 gene expression result in osteopenia [71]. In these animals, similarly to what occurs in human senile osteoporosis, the reduction in bone formation occurs faster than bone resorption. TghuRANKL (Tg5519) is a transgenic mouse overexpressing human RANKL resulting in the spontaneous development of osteoporosis similar to human pathology [71, 72]. The overexpression of huRANKL results in the spontaneous development of early onset osteoporosis characterized by lack of trabecular bone, increased osteoclastogenesis, increased bone remodeling, and decreased bone strength. Another model, already mentioned in this paragraph is $TRPV5^{-/-}$ mice that display hypercalciuria due to a primary renal failure to reabsorb calcium and hypervitaminosis D, leading to calcium hyperabsorption in the intestine and reduced bone mass [37, 73]. Other models are useful to evaluate osteocyte functions. They have been obtained,

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for example, by osteocyte-specific disruption of gap junction protein, *Gja1*. Osteocyte-specific *Gja1* conditional knockout mice show an increase in apoptosis of osteocytes [74].

Animal models for disused osteoporosis were also developed. Methods to reduce skeletal biomechanical loading include nerve, spinal cord, or tendon resections, casting, bandaging of one limb or suspension of both hindlimbs in rats. Immobilization-induced osteopenia/osteoporosis in rat skeletal model with the highly predictable pattern of bone loss and the hormone in plasma is significantly decreased [75].

Regarding osteogenesis, most of the studies have focused on osteogenesis imperfecta (OI), an autosomal dominant disorder caused by mutations in type I collagen, the most abundant protein of bone, skin, and tendon extracellular matrices. OI is characterized by increased bone fragility and low bone mass. Tansgenic mice expressing a premature stop codon or glycine substitution in the COL1A1 gene encoding the chains of type I collagen are good model for OI [76]. Very recently, the effect of high dose of hormones on bone density in OI patients has been reported [77]. Notably, new genes implicated in autosomal recessive forms of OI have been described and one of each is sphingomyelin phosphodiesterase (SMPD3), the gene encoding for neutral sphingomyelinase [78].

Some conflicting results are reported in literature, but the animal models developed so far have given valuable information on the pathogenesis of osteoporosis as well as on other pathological conditions of the skeleton and bone. The development of new animal models will help to better understand what have been poorly investigated in the past.

3. Vitamin D in nervous system physiology, neuroprotection, and neurogenesis

VDR is expressed in both neurons and glial cells (i.e., microglia, astrocytes, and oligodendrocytes) in different regions of the nervous system [3]. Vitamin D Response Element (VRE) response elements modulate gene expression. For example, it increases the expression genes codifying growth factors such as Nerve growth factor (NGF), glial cell line–derived neurotrophic factor (GDNF), NT3, and enzymes involved in the synthesis of neurotransmitters (tyrosine hydroxylase, tryptophan hydroxylase 2, and glutamate decarboxylase), whereas it decreases expression of voltage-dependent calcium channel [3, 79]. VDR is also expressed in the caveolae and induces nongenomic effects that include activation of cAMP-dependent protein kinase (PKA), Ca^{2+}/calmodulin-dependent protein kinase, phosphatidylinositol 3-kinase, and mitogen-activated protein kinase p38 leading to phosphorylation of neurofilaments, modulation of chloride, potassium, and voltage-dependent calcium channel in rat cortical neurons [80]. In addition, 25-hydroxylase and 1-a-hydroxylase activity are also found in the nervous tissue.

The combination of in vitro, ex vivo, and animal model data provides compelling evidence that $1,25(OH)_2D_3$ has a crucial role in neuronal proliferation, differentiation, neurotransmission, neuroplasticity, and neuroprotection. Increasing evidence derived from studies of $1,25(OH)_2D_3$ deficiency and from VDR polymorphisms implicates $1,25(OH)_2D_3$ as a candidate

in influencing susceptibility to a number of psychiatric and neurological diseases, such as schizophrenia, autism, Parkinson disease (PD), amyotrophic lateral sclerosis, epilepsy, Alzheimer disease (AD), and is especially strong for multiple sclerosis (MS) [8, 81, 82]. In epigenetic studies, maternal dietary deprivation of $1,25(OH)_2D_3$ has induced vitamin D deficiency (VDD) in rats prior to mating and maintained it during pregnancy. The $1,25(OH)_2D_3$-deficient rats showed modifications in brain morphology including increased overall brain size and larger lateral ventricles. Interestingly, some changes persist despite the addition of $1,25(OH)_2D_3$ to the diet of the pups. In adult life, these rats tend to demonstrate subtle alterations in learning and memory and impaired attentional processing [81]. Prenatal VDD induces similar alterations in fetal mouse brain morphology and mouse behavior [83, 84]. Notably, it has been reported that maternal $1,25(OH)_2D_3$ insufficiency during pregnancy in humans is also significantly associated with offspring's language impairment [85]. Interestingly, prenatal $1,25(OH)_2D_3$-depleted rats showed a significant impairment of latent inhibition, a feature often associated with schizophrenia [81].

The neuroprotective effect of $1,25(OH)_2D_3$ has been recently reported in cognitive decline of aging rats [86], and it has been extensively studied in the animal model of MS and the experimental allergic encephalomyelitis (EAE). The hormone prevents onset and reversibly blocks progression of clinical signs, but such a protective effect is absent in VDR knockout mice [81]. The effect of $1,25(OH)_2D_3$ might not be due exclusively to its neuroimmunomodulatory properties [81] since recently it has been reported that the hormone enhances neural stem cell proliferation and differentiation into neurons and oligodendrocytes, the myelinating cells of central nervous system [4, 87]. Neural stem cells constitutively express VDR, which can be upregulated by $1,25(OH)_2D_3$ [4].

$1,25(OH)_2D_3$ regulates the expression of many AD-related genes. It attenuates Aβ peptide accumulation by stimulating phagocytosis of Aβ peptide probably by modulating transcription of Toll-like receptors and cytokines together with enhancing brain-to-blood efflux transport by increasing P-glycoprotein expression [88].

Adult neurogenesis is limited to specific brain regions in the mammalian brain, such as the hippocampal dentate gyrus and the subventricular zone. Alterations in adult neurogenesis appear to be a common hallmark in different neurodegenerative diseases including PD and AD [89]. Therefore, factors that stimulate neurogenesis have been indicated as possible treatments of neurodegenerative disorders. The antiproliferative and prodifferentiating effects of $1,25(OH)_2D_3$ in neural cells were described more than 10 years ago [90, 91]. $1,25(OH)_2D_3$ decreases the expression of G1/S and G2/M cellular gatekeeper components, such as cyclins D1 and B1 [91, 92] and decreases the percentage of cultured hippocampal cells undergoing mitosis in conjunction with increases in both neurite outgrowth and NGF production [90]. Recently, ceramide kinase signalling pathway has been involved in the antiproliferative action of $1,25(OH)_2D_3$ human neuroblastoma cells [18]. Accumulated evidence indicates that $1,25(OH)_2D_3$ has complex effects on neurogenesis of neural stem cells. Cui et al. [93] have investigated the effect of Developmental vitamin D (DVD) deficiency on neuroprogenitor formation in the neonatal brain, and they have shown an increase in the number of neurospheres formed in cultures from the neonatal subventricular zone. Exogenous $1,25(OH)_2D_3$ added to

the culture medium reduced neurosphere number in control (in agreement with the putative antiproliferative effect of $1,25(OH)_2D_3$), but not in cultures from the deprived pups [93]. In contrast, neurogenesis in adult subgranular zone of the hippocampus is decreased [94]. In another model of $1,25(OH)_2D_3$ deficiency, Zhu et al. [95] have reported increased proliferation, but decreased survival of newborn neurons in the dentate gyrus of adult mice lacking $1,25(OH)_2D_3$, the 1α-hydroxylase knockout mice [95]. The different effects probably depend on the time window of exposition and/or the different sensibility to the hormone of distinct neurogenic niches.

The neuroprotective effect of high intake of $1,25(OH)_2D_3$ has been confirmed in some AD trials, but not in others [88]. It is not clear whether hypovitaminosis D triggers AD or it removes protection in the Central nervous system (CNS) against AD. However, the combination of antineurodegenerative drugs with $1,25(OH)_2D_3$ supplementation might be useful. Indeed, the supplementation of the combination nemantidine plus $1,25(OH)_2D_3$ has been shown to prevent cognitive decline more efficiently than that of the single compounds [96].

4. Sphingolipids

Sphingolipids (SLs) have long been regarded as inactive and stable structural components of the membrane, but they are biologically active molecules [11–14, 17, 19]. They are formed by the attachment of different polar headgroups at the primary alcohol group of a ceramide molecule. As reported in **Figure 1A** and **B**, there are two main pathways of Cer production: (1) *de novo* biosynthesis and (2) hydrolysis of sphingolipids, such as Sphingomielin (SM). Which of these pathways dominates depends on the cell type, stimulus, and developmental stage of the cell [97].

SLs are the main components of specific membrane platforms that function in membrane signaling and trafficking, named lipid rafts [98, 99]. The most studied SLs are the bioactive lipid S1P and Cer [14], even if in the last few years relevant role has been demonstrated also for ceramide 1-phosphate (C1P) [18, 99, 100] and Cer species [101].

S1P plays a crucial physiological function. In fact, it is present at different concentrations in plasma as well in tissues, constituting a gradient that drives the trafficking of various immune cells [102]. The S1P gradient is due to the constitutive activity of Sphingosine-1-phosphate lyase (SPL) in the cells, leading to a low concentration of S1P in most tissues (0.5–75 pmol/mg) and low-micromolar range in plasma (0.2–0.9 μM) where S1P, produced and released by platelets and erythrocytes, is complexed with albumin and lipoproteins, particularly High-density lipoprotein (HDL) [103]. Of note, it has been proposed that cell fate is regulated by the ratio between S1P and Cer/Sphingosine (Sph) described the first time by Cuvillier et al. [20]. (**Figure 1C**) S1P enhances cell growth and survival, whereas its precursors, Cer and Sph, are generally associated with cell growth arrest and death. However, recent results indicate that Cer species (short or long chain Cer) have different functions [104].

S1P has a peculiar mechanism of action. In fact, S1P, produced inside the cell, can act as intracellular mediator or can be exported outside by the putative transporter Spinster 2, spns2 [105, 106]. Outside the cells, S1P can act as ligand of specific G-protein-coupled receptors. Five

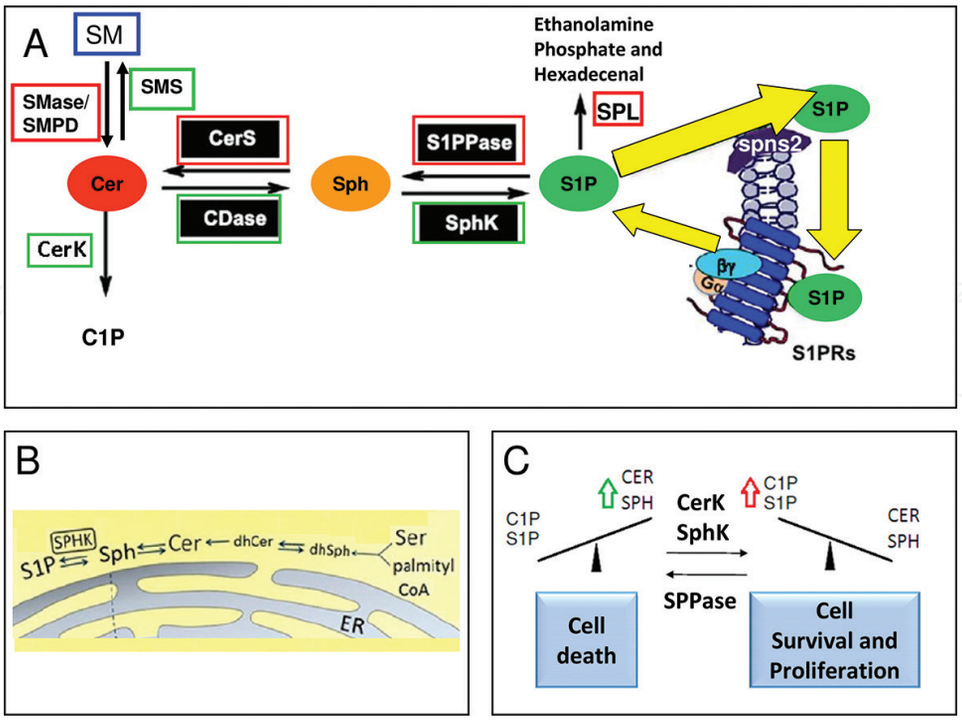


Figure 1. Schematic representation of sphingolipid metabolism pathways and rheostat model. (A) Cers are produced from Sphyngomyelin (SM) by activation of sphingomyelinase (SMase/SMPD) that produces Cer and phosphocholine. The reverse reaction is catalyzed by SM synthase (SMS). Ceramides can also be phosphorylated by ceramide kinase (CerK) to form ceramide-1-phosphate (C1P). S1P is derived by Cer deacylation by ceramidases (CDase) to Sph followed by its phosphorylation by Sph kinases (SphK). The degradation of S1P requires S1P phosphatases activity that produces Sph, and by S1P lyase (Spl) that produces hexadecenal and phosphoethanolamine. (B) *De novo* synthesis of SLs. The *de novo* synthesis is initiated at the cytosolic membranes of the endoplasmatic reticulum and in mitochondria, via the condensation of l-serine with palmitoyl-CoA to form 3-keto-dihydrospingosine, and it is catalyzed by serine palmitoyltransferase (SPT). The product of SPT is reduced by a hydroxyl by 3-keto-dihydrosphingosine reductase in a NADPH-dependent manner producing dihydrosphingosine that is subsequently N-acylated to dihydroceramide (dhCer) by ceramide synthases (CerS). In mammals, CerS is encoded by six distinct genes, and each enzyme has a distinct, but overlapping acyl CoA preference. Dihydroceramide desaturase converts the DHSph backbone within Cer into Sph. (C) Sphingolipids rheostat model.

S1P specific named EDG/S1PR$_{1–5}$ have been described to mediate S1P signaling [11, 13, 14, 19]. S1P1, S1P2, and S1P3 show broad tissue gene expression, while S1P4 shows gene expression primarily in immune system cells, and S1P5 is primarily expressed in the spleen (natural killer cells and other lymphocytes) and central nervous system [107]. Regarding the intracellular S1P effects, the bioactive lipid induces calcium release from the ER, alters the function of intracellular proteins, such as E3 ligase activity of TNF receptor associated factor 2 (TRAF2), binds the mitochondrial protein, and regulates mitochondrial assembly and function. When produced in the nuclei, S1P modulates gene expression inhibiting histone deacetylases [107].

4.1. Sphingolipids in bone physiology

Various SLs play a crucial role in the development of normal skeletogenesis acting in three different skeletal cell types: chondrocytes in cartilage and osteoblasts and osteoclasts in bone. Abnormal tissue development of genetically modified animal models, such as mouse lacking

sphingomyelin phosphatase 3 (SMP3) also named sphingomyelinase 2 (SMase2), have been reported [108]. As described in the first part of this chapter, SMase are a family of different isoenzymes. Among them, the nSMSase2/SMPD3 is largely expressed in bone and cartilage, and it is involved in Cer-mediated signaling events [109]. In other studies, the role of SLs in bone have been performed by using synthetic analogues of ceramide (i.e., C2-Cer), specific inhibitors of the rate limiting enzyme (i.e., SphK), and agonists and antagonists that mimic or inhibit, respectively, the function of S1P, as ligand, of specific receptor subtypes. In this paragraph, we examine the involvement of Sph/S1P and Cer/C1P axis in the remodelling and physiology of bone.

4.1.1. S1P—sphingosine

Several studies demonstrate that osteoblasts at different stages of differentiation may respond differently to SLs [110]. S1P has been shown to promote the proliferation of rat primary chondrocytes, whereas in preosteoblast MC3T3-E1, it negatively regulates the synthesis of the osteoblast marker osteocalcin [111, 112]. Notably, S1P can be released by osteoclasts [113] and secreted S1P can promote bone formation by enhancing the differentiation of osteoblast precursors to functional osteoblasts and by the recruitment of MSCs leading to high bone mass phenotype [114]. Recently, Keller et al. [115] report that the release of S1P by osteoclasts is regulated by calcitonin. S1P induces the upregulation of osteoptotegerin and osteoblast differentiation markers in two osteoblast-like cell lines promoting the translocation of β-catenin [116].

In the same cell type, Sph and S1P induce intracellular calcium release [117, 118] and in rat osteoblasts and in human osteosarcome cells; both sphingoids prevent the apoptotic process elicited by serum deprivation [119]. A functional cross talk exists between S1P and platelet-derived growth factor (PDGF) signaling. In fact, S1P limits, whereas PDGF promotes the migration of preosteoblasts, and it is the balance between these two bioactive molecules that allow only the differentiated osteoblasts to reach the site of bone formation. Very recently, it has been confirmed that in humans the detrimental effects of S1P on bone metabolism depend on the S1P gradient between blood and bone marrow cavity and on S1P receptor subtypes, mainly S1P1 and S1P2, play a crucial role in this control [112, 120]. In particular, S1PR1 exerts positive chemotaxis action on an S1P gradient, whereas S1PR2 counteracts this positive effect [121]. The deletion of S1PR2 led to moderate osteopetrosis by affecting the homing of osteoclast precursors into bone [112, 120]. Higher circulating S1P levels are associated with lower bone mineral density, higher levels of bone resorption markers, and higher prevalence and severity of osteoporotic vertebral fracture in Koreans [122]. Therefore, S1P appears to be an important osteoclast-derived anabolic factor that couples bone resorption to bone formation, but it preferentially influences bone resorption rather than bone formation in humans.

Notably, increase in the secretion of BMP-2, OPN, and osteocalcin as well as highest extracellular matrix mineralization and osteonodules formation were observed when MSCs are cultured on thin titania dioxide coatings (TiO_2) on stainless steel substrate doped with S1P [TiO_2/S1P(CII_m)] [123]. Therefore, S1P, also in an appropriate combination with other sphingoid, such as C1P, may find wide application in regenerative medicine, particularly in bone regeneration with the use of MSCs [123].

4.1.2. C1P—ceramide

Abnormal cartilage development and bone mineralization defects are observed in animal models in which the enzyme inactivation is generated by gene targeting (Smpd3−/− model) or chemically induced deletion in the Smpd3 locus (fro/fro model) [124, 125]. Several evidence underlines the importance of phosphocholine in bone mineralization [126]; however, at present no experimental data directly links Cer or choline to Extracellular matrix (ECM) mineralization. Studies in animal models are in some way in conflict with studies performed in cell culture: the inhibition of nSMase2/SMPD3 by GW4869 accelerates the mineralization of chondrogenic ATDC5 cultures [127], whereas either the fro/fro mice or mice lacking choline kinase presents an expansion of hypertrophic zone likely due to a delay in apoptosis of hypertrophic chondrocytes [128]. The role of Cer in bone tissue has been examined by other approaches leading also to conflicting results, such as short-chain synthetic cell-permeable forms of Cer, preferentially C-2 Cer [129]. In fact, it is not clear whether exogenous Cer has the same cellular targets as the endogenous species and the dose used in cell culture may not relate to the endogenous levels found under physiological or pathological conditions. Therefore, it is reported that Cer may be able to promote cell apoptosis or cell survival, and it may depend on the dose and cell type used. The treatment of cells with high-dose of C-2 Cer (1–10 μM) leads to apoptosis of mouse primary osteoblasts. Contrary, the low-dose treatment (10 time less or more) of C-2 Cer promotes an antiapoptotic effect in other cells [130]. Regarding cell types, C-2 Cer treatment leads to increased apoptosis in both osteoblasts and chondrocytes, while there was no effect on the apoptosis of rabbit osteoclasts [131]. Recently, it has been demonstrated that short-chain C-6 Cer induces anti-osteosarcoma activity in vitro and in vivo [132]. In other studies, Cer has been shown to be mitogenic in preosteoblast MC3T3-E1 cells [133]. Recently, Cer species, in particular long-chain Cer (C-22 and C-24 Cer), have been demonstrated to mediate the proapoptotic effect of sodium nitroprusside, a nitric oxide donor, in MC3T3-E1 cells [134], suggesting that the controversial results may also be due to different levels of Cer species. Detectable increase of endogenous Cer levels is observed when cells are induced to apoptosis by TNF-alpha [135]. This upregulation leads to the modulation of NF-kappa B localization and function [136]. Different intracellular signaling pathways are involved in osteoblast death and survival: Cer induces osteoblast apoptosis through protein phosphatase 1 and protein kinase C (PKC) δ [137, 138], whereas the ability of Cer to promote osteoblast survival is prevented by PKC ζ inhibitor. At present, it is not clear whether Cer acts up- or downstream to or independently of caspases [139]. Interestingly, in mice lacking the enzyme that converts dihydroceramide to ceramide (dihydroceramide desaturase 1) Cer reduction does not lead to bone defects, indicating that the maintenance of Cer levels is not essential for normal bone mineralization. Recently, it has been found a role also for C1P in the osteogenesis of multipotent stromal cells derived from MSCs. C1P can affect the growth and expanded intercellular connections, thereby, increasing the proliferative activity, acting in opposite manner of S1P. Therefore, it has been suggested that an appropriate combination of C1P and S1P may be a useful strategy in bone regeneration with the use of MSCs [123].

4.2. Sphingolipids in nervous system physiology

SLs are particularly abundant in the central nervous system. Mutations in genes coding for enzymes involved in their metabolism cause sphingolipidosis many of which show alterations

in the nervous system (reviewed by Sabourdy et al. [140]). In addition, modifications in SL metabolism are found in neurodegenerative diseases, such as AD, PD, Huntington disease (HD), MS, and major depression [141–146]. Various SLs are crucial in regulating neural physiological functions, including cell survival, apoptosis, differentiation, inflammation, excitability, and neurotransmitter release [15, 137–149].

4.2.1. S1P—sphingosine

Like Cer, Sph acts as a proapoptotic signal as well as an inhibitor of several enzymes such as protein kinase C (PKC), phospholipase D, and of the transcription factor SF-1 [15, 150]. Sph also directly modulates voltage-activated calcium channels in pituitary cells and several components of the melastatin-like transient receptor potential channel subfamily, such as Transient receptor potential cation channel subfamily M member 3 (TRPM3) [151, 152]. Sph alters the integrity of membranes and induces the release of lysosomal cysteine proteases, such as cathepsins and of cytochrome *c*, which in turn activates the intrinsic pathway of apoptosis. Increasing evidence indicates that Sph regulates vesicle fusion and trafficking and, therefore, the strength and reliability of synaptic transmission [153, 154]. In addition, Sph is a competitive antagonist of the type 1 cannabinoid receptors (CB1Rs) [155] and possibly it contributes to SL regulation of nociception [156]. S1P modulates survival, proliferation, differentiation, cell migration, calcium homeostasis, neurite retraction, angiogenic vascular maturation, and cytoskeleton dynamics [13, 107, 149]. The bioactive lipid can also induce neuroprotection through many mechanisms that include production of growth factors, decrease of oxidative stress, increase of Mitogen-activated protein kinase (MAPK) activation, activation of PI3K/AKT pathway, modulation of antiapoptotic proteins, pigenomic effects by direct inhibition of deacetylases, and affecting mitochondrial functionality (for a recent review see Ref. [149].

S1P receptors are expressed in CNS cells (neurons, oligodendrocytes, astrocytes, and microglia) and their expression levels change during development. The role of S1PR depends on cell types and on their different expression and localization during development or following stimulation. The studies of knockout mice with deficits in both Sphk1 and Sphk2 highlight the importance of S1P in the development of the nervous system. These mice show severely disturbed neurogenesis, including neural tube closure and angiogenesis, and they die at an early embryonic stage [157]. Migration of neural stem progenitor cells toward injury sites is promoted by S1P via S1P1 [158] and inhibited on the oligodendrocyte progenitor cells via S1P5 [159]. S1P receptors are expressed in CNS cells (neurons, oligodendrocytes, astrocytes, and microglia) and their expression levels change during development. Analogously to the double SphK knockout, the S1P1 knockout mouse shows altered neurogenesis and angiogenesis, while the knockout of the other receptors have less severe consequences. Maclennan et al. [160] reported that mice lacking S1P2 show significant increase in excitatory postsynaptic potentiation, resulting in spontaneous seizures. The role of S1PR depends on their different expression and localization during development or following stimulation. For example, activation of S1P5 causes activation of Rho and retraction of processes in immature oligodendrocytes and survival in mature cells [161]. NGF induces differentiation in PC12 cells through a relocalization of S1P receptors; S1P1 that induces neurite growth is expressed at the plasma membrane, while S1P2 is internalized [162] becoming unable to cause cell rounding and loss of neurites. S1P and neurotrophic factors have mutual effects on expression of each other:

NGF and GDNF are able to stimulate S1P generation, and S1P increases GDNF production by astrocytes [144]. S1P plays different roles in synaptic transmission. It increases glutamate release in hippocampus via S1P3 [163], it enhances excitability in rat sensory neurons trough S1P1 and S1P3 [155, 158, 164], and it is involved in the recruitment of vesicles in the presynaptic membrane [165] and also in endocytic membrane trafficking [166].

4.2.2. C1P—ceramide

Cer regulates cell growth, differentiation, apoptosis, inflammation, exosome release, and neural excitability [15, 157, 158, 167, 168]. Accumulating evidence indicates that different Cer species might have different functions [169]. Cer 18:0 is synthesized by CerS1, an enzyme abundant in the brain, and appears to have a protective role [167, 168]. Serum deprivation that increases apoptosis in embryonic hippocampal cells increases Cer 16:0 and decreases Cer 24:0 content [27]. Cer has been involved in synaptic regulation and plasticity [170, 171]. For example, Cer is able to increase dopamine release and uptake [172] and to modulate excitatory postsynaptic currents by controlling the insertion and clustering of NMDA receptors [173]. Moreover, SMase2 inhibition delays formation of spatial memory in mice [174]. Regarding inflammation, astrocytes display increased Cer following ischemia/reperfusion leading to generation of pro-inflammatory cytokines [175]. Recently, it has been reported that Cer induces ciliogenesis, a critical step in differentiation in embryonic stem cells and neural progenitors [176]. CerK, the enzyme generating C1P, was first observed in brain synaptic vesicles [177] and found to be highly expressed in brain [178] suggesting a role of C1P in neurotransmitter release. C1P induces proliferation or survival in several types of cells including macrophages and fibroblasts [179] while inhibition or downregulation of CerK decreases proliferation in human neuroblastoma cells [18]. C1P plays a regulatory role in inflammation since it directly binds and activates α-type cytosolic phospholipase A2 stimulating arachidonic acid release [180]. Recently, it has been demonstrated that C1P plays an important role in recruitment of stem/progenitor cells to damaged organs [181]. Whether C1P is also released from the injured nervous system or whether it induces migration in nervous stem cells is unknown.

5. Cross talk between vitamin D and sphingolipid metabolism: a potential role in Alzheimer's disease

$1,25(OH)_2D_3$ and SL metabolism cross talk at different levels. For example, $1,25(OH)_2D_3$ modulates the expression of genes involved in S1P degradation, such as Sphingosine-1-phosphate phosphatase 2 (SGPP2) [182] and of growth factors involved in differentiation and neuroprotection, such as NT-3, Brain-derived neurotrophic factor (BDNF) and ciliary neurotrophic factor (CNTF) [3, 4]. On the other hand, many neuroprotective actions of $1,25(OH)_2D_3$ have been reported to be due to stimulation of SphK and increased levels of S1P [183, 184]. In addition, $1,25(OH)_2D_3$ also is able to decrease the expression of CerK, the enzyme that generates C1P [18]. C1P activates directly Secretory PLA2 enzymes (sPLA2) [180], producing arachidonic acid that can be further metabolized to proinflammatory mediators. C1P and S1P have a crucial role in migration. C1P is released from damaged cells and chemoattracts bone marrow-derived

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multipotent stromal cells, endothelial progenitor cells, and very small embryonic-like stem cell. The migration of osteoclast precursors is controlled by S1P and, recently, it has been found that $1,25(OH)_2D_3$ reduces the expression of the chemorepulsive receptor S1P2 on circulating precursors [185]. AD is a neurodegenerative disorder of the central nervous system and the most common form of dementia [186]. The pathogenic hallmarks of AD include extracellular amyloid-containing plaques, intracellular neurofibrillary tangles consisting of hyperphosphorylated tau protein and death of cholinergic neurons of the basal forebrain. Amyloid plaques are mainly formed by aggregated amyloid beta peptide (Aβ). Alterations in the enzymes involved in SL metabolism and content have been observed in brains and cerebrospinal fluid of AD patients [187–192], leading to an increase of Cer and loss of S1P. Many studies in culture cells and animal models have demonstrated that Aβ affects SL metabolism. For example, Aβ42 directly binds and activates nSMase in vitro decreasing SM content [193]. In addition, Aβ can activate both neutral and acidic SMases through increased ROS accumulation via Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation and glutatione (GSH) depletion [194]. Increased Cer could then induce apoptosis [186, 195, 196]. In addition, Cer increases stability of β-amyloid precursor protein cleaving enzyme (BACE1) activity [197], while S1P binds to and increases proteolytic activity of BACE1 [189] and SM decreases Aβ production by inhibition of the γ-secretase [193]. Summarizing, some SLs might be protective by enhancing Aβ clearance or decreasing Aβ production, others increase Aβ toxicity or Aβ oligomerization and at the same time, amyloid precursor protein processing also affects lipid metabolism, resulting in complex regulatory feed-back cycles, which appear to be dysregulated in AD. It is worth noting that recently, a novel mechanism of Cer-enriched exosomes released by Aβ-treated astrocytes has been proposed to be responsible for Aβ-induced apoptosis [167].

The neuroprotective action of SL analogues, such as FTY720, has been tested in some neurodegenerative diseases. FTY720 is a prodrug that is converted to an analogue of S1P when phosphorylated by SphK. It has been approved for the treatment of multiple sclerosis (MS) and acts both as an immunomodulatory drug and on different cells of the CNS (neurons, astrocytes, oligodendrocytes, and microglia), all of which express S1P receptors [198]. Administration of FTY720 in a rat model of AD obtained by injection of Aβ decreases death in hippocampus and cortex and increases memory compared with control rats [199, 200]. FTY720 decreases production of Aβ in cultured neuronal cells [189]. Increasing evidence derived from epidemiological studies indicate that $1,25(OH)_2D_3$ deficiency and VDR polymorphisms influence susceptibility to AD [201], whereas Aβ may disrupt the hormone-VDR pathway and cause defective utilization of $1,25(OH)_2D_3$ by suppressing the level of the VDR and elevating the level of 24OHase [202]. In addition to neuroprotective effects involving calcium, Reactive Oxygen Species (ROS), and inflammation, $1,25(OH)_2D_3$ is able to exert other specific effects important for AD, by regulating the expression of many AD-related genes. It attenuates Aβ peptide accumulation by stimulating phagocytosis of Aβ peptide probably by modulating transcription of Toll-like receptors and cytokines together with enhancing brain-to-blood efflux transport by increasing P-glycoprotein expression and likely by altering Amyloid Precursor Protein (APP) processing [88] and prevents the acetylcholine defect by increasing the activity of choline acetyltransferase (thus acetylcholine synthesis) in the brain [203]. Part of VDR is also located in lipid microdomains in the nuclear membrane [2], and

this localization is modified by altering SL metabolism and has been associated with embryonic hippocampal cell differentiation. Neuroprotective actions of SLs, in particular S1P, and $1,25(OH)_2D_3$ include many common effectors such as calcium regulation, synaptic modulation, growth factor expression, regulation of inflammation, etc., but whether $1,25(OH)_2D_3$ and SLs, in particular S1P, could act synergistically on neuroprotection and/or neurogenesis in AD is still unknown and deserves further investigation. A study in our lab indicates that the cross talk between SLs and $1,25(OH)_2D_3$ leads to a specific balance between neurodegeneration/neuroprotection in neuronal cells [204]. The neuroprotective effect of high intake of $1,25(OH)_2D_3$ has been found in some AD trials, but not in others [88]. It is not clear whether hypovitaminosis D triggers AD or it removes protection in the CNS against AD. However, the combination of antineurodegenerative drugs with $1,25(OH)_2D_3$ supplementation might be useful. In fact, the supplementation with nemantidine plus $1,25(OH)_2D_3$ has been shown to prevent cognitive decline more efficiently than that with the single compounds [96]. Regarding MS, very limited evidence suggests a potential benefit of $1,25(OH)_2D_3$ supplementation for the prevention of MS and this needs to be further verified by future studies [3].

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