CNS Neuroscience & Therapeutics

REVIEW

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Vitamin D as a Potential Therapy in Amyotrophic Lateral Sclerosis

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Keywords
Amyotrophic lateral sclerosis; Apoptosis; Calcidiol; Calcitriol; D3; G93A mice; Excitotoxicity; Inflammation; Neurodegenerative disease; Neuromuscular disease; Motor neuron death; Oxidative stress; Vitamin D.

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Received 30 October 2012; revision 30 September 2013; accepted 11 October 2013

SUMMARY

Vitamin D has been demonstrated to influence multiple aspects of amyotrophic lateral sclerosis (ALS) pathology. Both human and rodent central nervous systems express the vitamin D receptor (VDR) and/or its enzymatic machinery needed to fully activate the hormone. Clinical research suggests that vitamin D treatment can improve compromised human muscular ability and increase muscle size, supported by loss of motor function and muscle mass in animals following VDR knockout, as well as increased muscle protein synthesis and ATP production following vitamin D supplementation. Vitamin D has also been shown to reduce the expression of biomarkers associated with oxidative stress and inflammation in patients with multiple sclerosis, rheumatoid arthritis, congestive heart failure, Parkinson's disease and Alzheimer's disease; diseases that share common pathophysiologies with ALS. Furthermore, vitamin D treatment greatly attenuates hypoxic brain damage *in vivo* and reduces neuronal lethality of glutamate insult *in vitro*; a hallmark trait of ALS glutamate excitotoxicity. We have recently shown that high-dose vitamin D_3 supplementation improved, whereas vitamin D_3 restriction worsened, functional capacity in the G93A mouse model of ALS. In sum, evidence demonstrates that vitamin D, unlike the antiglutamatergic agent Riluzole, affects multiple aspects of ALS pathophysiology and could provide a greater cumulative effect.

doi: 10.1111/cns.12204

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS; Figure 1), also known as "Lou Gehrig's disease," is a fatal neurodegenerative disease of the motor cortex, brain stem and spinal cord; responsible for the destruction of upper and lower motor neurons causing paralysis [1]. Currently, at least 26 mutant genes are known to cause ALS in humans (Table 1). Of the known genetic defects, the most studied of these is mutant Cu/Zn superoxide dismutase (SOD1, comprising approximately 20% of all known inherited mutations [2]). Most ALS patients will begin to experience symptoms usually manifesting as weakness in the limbs, progressing to affect manual dexterity and gait, eventually losing most voluntary control [1]. Death is eventually caused due to respiratory failure with a median survival rate of 3–5 years after the onset of symptoms [3]. The only generally accepted treatment for the disease is the administration of the antiglutamate drug Riluzole, which is by far the most prescribed therapy for ALS [4]. Daily 100 mg oral consumption of the drug is reported to prolong the median survival of patients by approximately 2–3 month and increase the likelihood of survival in the first year by 9% [5].

Rationale for Vitamin D as a Therapeutic in ALS

Amyotrophic lateral sclerosis shares pathophysiological similarities with various diseases such as congestive heart failure, rheumatoid arthritis (RA), multiple sclerosis (MS), Alzheimer's disease (AD), and Parkinson's disease (PD). These similarities include oxidative stress, inflammation, neurodegeneration, mitochondrial dysregulation, and apoptosis [6–11]. Evidence suggests that vitamin D ameliorates these pathophysiologies in animal disease models and human patients [7–20], and may therefore be able to attenuate the sequelae of ALS (Figure 2). Recent studies have shown that high-dose vitamin D_3 (D_3) supplementation improves paw grip endurance and motor performance in the G93A mouse model of ALS [21,22]. In contrast, D_3 restriction hastens the decline in paw grip endurance and motor performance postdisease onset in the same

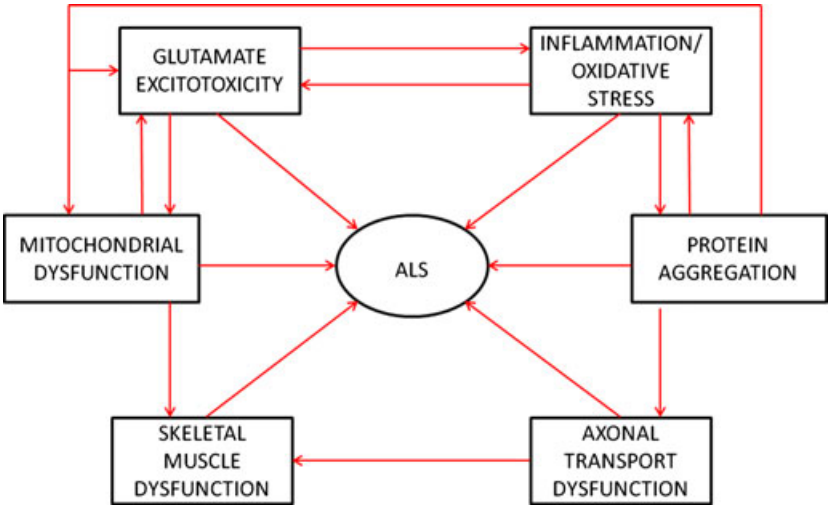

Figure 1 Schematic outlining the multifaceted nature of ALS pathology. ALS, amyotrophic lateral sclerosis.

Table 1 Known mutated genes known to cause amyotrophic lateral sclerosis

Gene protein product	Normal protein function	References
Superoxide dismutase 1	Antioxidant	2,150
Alsin	GTPase regulation	151
ALS3	Unknown	152
ALS7	Unknown	153
Senataxin	RNA processing	154
Vesicle-associated membrane protein/synaptobrevin-associated membrane protein B	Intracellular vesicle trafficking	155,156
Angiogenin	Angiogenic regulation	157,158
TAR DNA binding protein-43	Transcriptional regulation	159
Fused in sarcoma	Transcriptional regulation	160,161
Dynactin p150 subunit	Axonal transport	162
Spatacsin	Axonal transport	163
Ubiquilin 2	Protein degredation	164
SIGMAR 1	Receptor	165
C9orf72	Unknown	166
Peripherin	Neurofilament subunit	167
Valosin-containing protein	Intracellular vesicle trafficking	168
Ewing sarcoma breakpoint region 1	RNA processing	169
Optineurin	RNA processing	170
Ataxin 2	RNA processing	171
Neurofilament heavy chain	Cell structure	172
Charged multivesicular body protein 2b	Intracellular vesicle trafficking	173
Phosphatidylinositol 3,5-bisphosphate 5-phosphatase	Intracellular vesicle trafficking	174
D-amino acid oxidase	Protein metabolism	175
Profilin 1	Cell structure	176
Sequestosome	Protein metabolism	177
TATA-binding protein associated factor 15	RNA binding protein	178

mouse model [23]. Indeed, a very recent ALS clinical study concluded that D3 supplementation reduced the decline in the revised amyotrophic lateral sclerosis functional rating scale (ALSFRS-R) score versus non-supplemented patients [24]. These findings are supported by a retrospective study, which also found that patients with vitamin D deficiency (serum calcidiol > 25 nM) had a 6 fold higher rate of death compared to patients with high vitamin D status (serum calcidiol > 75 nM) [25]. In sum, there is substantial support for vitamin D as a potential therapeutic in ALS.

Vitamin D as Related to ALS Pathology

Human Vitamin D Studies Related to ALS

Inflammation and Oxidative Stress

The chronic, feed-forward cycle of glial cell activation leading to inflammatory cytokine generation, microglial proliferation, and neurotoxicity in ALS constructs an event referred to as "neuro-inlammation" [26]. Tumour necrosis factor-alpha (TNF-α), a potent inflammatory cytokine, induces apoptosis and contributes

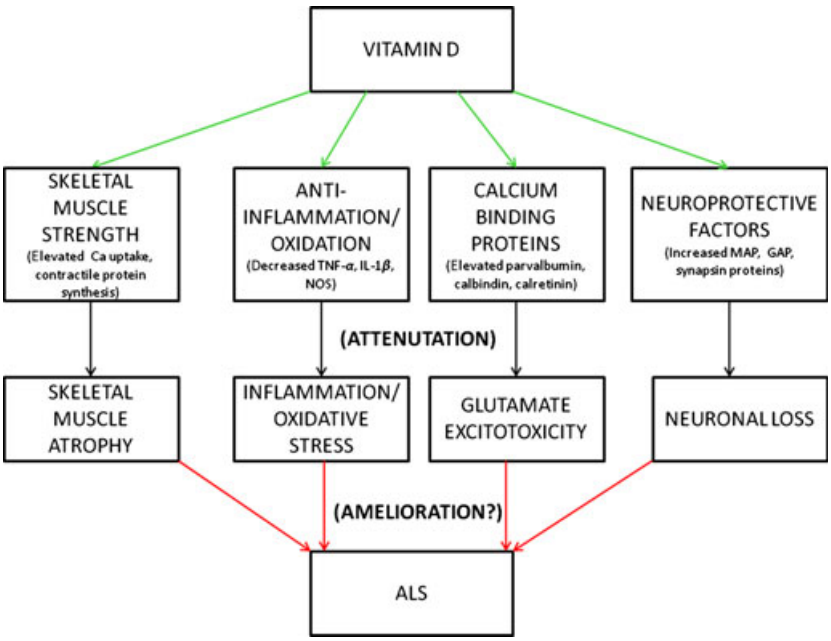

Figure 2 Schematic of the potential amyotrophic lateral sclerosis (ALS) pathophysiologies modulated by vitamin D and the possible subsequent mitigation of ALS. (TNF-α, tumor necrosis factor- α; IL-1β, interleukin-1β; NOS, nitric oxide synthase; MAP, microtubule-associated protein; GAP, growth-associated protein).

to oxidative stress by activating microglia [26]. This important cytokine is found in elevated amounts in G93A mouse spinal cords [27] and human ALS patient serum [28,29]. Following administration of thalidomide and lenalidomide (agents used to treat some cancers that also inhibit TNF-α expression) starting at 30 day, G93A mice exhibited significant improvements in multiple outcome measures when compared with saline-treated controls including: improvement in motor performance between 98 and 155 day of age, attenuation in weight loss from 70 day of age, and extention of mean survival by 12–18.5% [30]. Interleukin-1β (IL-1β) is also involved in inflammatory-mediated damage in ALS where it stimulates the destruction of cellular proteins via transcription of apoptotic enzymes including caspase 1 and caspase 3 [31]. In line with this, human ALS spinal cords exhibit high concentrations of IL-1β [32]. Matrix metalloproteinases (MMP) are capable of degrading all components of the extra cellular matrix [33] and MMP-9, activated by TNF-α and IL-1β [34], is also implicated in ALS pathology as well as other related neurologic conditions such as AD, PD, stroke, and spinal cord trauma [35–41].

In 174 patients with kidney disease, serum $1,25(OH)_2D_3$ negatively correlated with urinary MCP-1 (a marker of inflammation; $r = -0.342$), renal scarring ($r = -0.546$), and macrophage infiltration ($r = -0.537$) [42]. Healthy male mononuclear cells insulted with lipopolysaccharide (LPS) exhibited a 64% and 59% reduction in TNF-α and IL-1β, respectively, in tandem with an increase in median blood $25(OH)D_3$ from 43 nM in winter to 89 nM in summer [43]. Healthy Canadian natives supplemented with 1000 IU D_3/day for 8 months exhibited a 16 fold decrease in IL-1β mRNA in cultured macrophages exposed to tuberculosis lipoprotein versus baseline [42]. In a randomized, double-blind, placebo-controlled trial in patients with congestive heart failure (characterized by a reduced cardiac ejection fraction, cardiac hypertrophy, and increased pro-inflammatory cytokines, particularly TNF-α [7]), 2000 IU D_3/day for 9 months increased median expression of the anti-inflammatory IL-10 by 43% versus baseline [7]. Median TNF-α levels increased 12% in the placebo group, but did not change significantly from baseline in the D_3 group [7]. A more recent randomized, double-blind, placebo-controlled trial administered a single dose of 250,000 IU D_3 to cystic fibrosis patients (characterised by chronic lung infection and inflammation) and observed a 50% reduction in TNF-α expresstion versus placebo [45]. Peterson et al. [46] found that mean serum TNF-α concentration was 35% lower with high (0.79 pg/mL) versus low (1.22 pg/mL) UV exposure, and that serum $25(OH)D_3$ was negatively correlated with serum TNF-α ($r = -0.25$) in healthy women. Indeed, a 2010 US prospective database study involving 41,497 men and women (age, 55 ± 21 years) showed that those with serum $25(OH)D_3$ <37.5 nM had a 45%, 45%, and 78% greater likelihood of developing coronary artery disease, peripheral vascular disease, and stroke, respectively, versus those with levels >75 nM [47]. Human patients with rheumatoid arthritis (a chronic inflammatory autoimmune disease) exhibit low serum levels of $25(OH)D_3$ and $1,25(OH)_2D_3$ [48], suggesting that vitamin D plays a role in the disease. In a 3-month clinical trial involving 19 patients with RA treated with standard antirheumatic drugs, daily oral 2 μg doses of alphacalcidiol (a vitamin D analog) improved clinical measures such as Ritchie Articular Index, and improved biological measures such as lymphocyte proliferation and apoptosis in 89% of the patients. These human studies suggest the possibility that vitamin D supplementation could reduce ALS neuroinflammation and oxidative stress.

Muscle

Amyotrophic lateral sclerosis is characterized by "amyotrophy," indicative of the muscle wasting due to the denervation of muscle fibers and their degenerating motor neurons [1]. "Lateral sclerosis" refers to the hardening of ventral and lateral corticospinal tracts as these areas are progressively replaced by gliosis (the pro-

cess involving the accumulation and death of neuroglia at sites of damage in the central nervous system resulting in scarring) [1]. A phenotypic hallmark of ALS is the atrophy of skeletal muscle fibres which become denervated as their corresponding motor neurons degenerate [49].

The nuclear VDR is involved in regulating a large number of genes (up to 5% of the total human genome [50]) and is indeed expressed by human muscle tissue [51]. A cross-sectional study involving 127 Dutch elderly aged >65 years found a modest association between serum $25(OH)D_3$ and appendicular lean mass ($\beta = 0.012$), as well as physical performance ($\beta = 0.020$) [52]. After an acute bout of intense exercise, serum $25(OH)D_3$ was inversely correlated with the postexercise muscle weakness experienced by one leg versus the other nonexercised control leg immediately after exercise ($r = -0.71$), as well as at 48 h ($r = -0.67$) and 72 h ($r = -0.72$) postexercise [53]. Data from 4100 ambulatory $\geq$ 60 year-old adults demonstrated a dose–response relationship between serum $25(OH)D_3$ levels and the ability to walk 8 ft. and sit-to-stand test; whereby those in the highest $25(OH)D_3$ quintile scored 6% and 4% higher, respectively, versus the lowest quintile [54]. Supplementing 75–88 year-old men and women (serum $25(OH)D_3$ <50 nM) with 800 IU D_3/day in double-blind, RCTs decreased the risk of falls by 27–72% [55–58]. Further, 63–99 year-old women supplemented for 3 months with 800 IU D_3/day + 1200 mg/day calcium improved their musculoskeletal function (knee flexor and extensor strength, grip strength, and the timed up-and-go test) by 4–11% versus baseline, in contrast to the −4% to 1% change in strength demonstrated by the control group supplemented only with calcium [55]. This increase in strength probably contributed to the 49% lower rate of falls experienced by the group supplemented with D_3 and calcium versus only calcium. In contrast, 251 healthy adults aged 18-50 years given 1000 IU/day or placebo for 4 months did not improve in the chair-rising test, hand grip strength, or jump height versus baseline [59]. Separately, treating vitamin D-deficient approximately 70 year-old women with alphacalcidiol (vitamin D analog with a negligible calcemic effect) for 6 months increased isometric knee extensor strength by 13% and total walking distance traveled over 2 min by 10% as compared to baseline [60]. In hemodialysis patients, $1,25(OH)_2D_3$ supplementation increased three repetition-maximum knee extension, knee extension peak torque, and ankle dorsiflexion by 21–38% versus placebo [61]. These gains in strength were concurrent with an 11% and 15% increase in *tibialis anterior* and thigh cross-sectional areas, respectively. In support, 21 mobility-limited women aged $\geq$ 65 years given 4000 IU/day increased intramyonuclear VDR by 30% and muscle fibre size by 11% [62]. Most relevant, vitamin D_3 supplementation at 2000 IU/d for 9 months was recently shown to reduce the decline in the ALSFRS-R score [24]. A separate study supported these findings retrospectively by associating low vitamin D status with poor ALS prognosis according to the ALSFRS-R [63].

Neurodegeneration and Neuroprotection

Excessive glutamate, the main excitatory neurotransmitter in the CNS [64], is an inherent part of ALS pathology. Excessive glutamate release by the presynaptic neuron into the synaptic cleft and/or impaired glutamate removal by EAAT2 transposters located on synapse-enveloping astrocytes in ALS causes prolonged activation of postsynaptic receptors, resulting in the influx of excessive quantities of sodium and calcium into the cell, inducing free radical production [65]. Due to a limited amount of intracellular calcium-buffering proteins, motor neurons are vulnerable to excessive calcium concentrations [66]. Mice that highly express intracellular calcium-buffering proteins are more resistant to excitotoxicity and exhibit lower concentrations of intracellular calcium following AMPA receptor stimulation [66]. Poor clearance of glutamate from the synapse can also, in part, be responsible for excitotoxicity due to a decline in the number of functional astrocytic glutamate reuptake transporters, either due to a decrease in the number of transporters or an increase in dysfunctional/nonfunctioning transporters [64]. Increases in intracellular calcium can induce mitochondria to generate free radicals which escape the cell and further compromise the ability of synaptic glutamate reuptake by glutamate transporters [65]. The high influx of calcium can also cause neuronal mitochondria to swell, opening the mitochondrial permeability transition pore and releasing proapoptotic factors [67].

Support for the role played by vitamin D in the nervous system is strengthened by the discovery of its machinery in the postmortem human brain [68] and nuclear vitamin D uptake in the spinal cord [69]. VDR is very strongly expressed in the CA1 and CA2 regions of the brain, but with a lower amount in the CA3 region of the hippocampus, whereas 1α-OHase is very strongly and evenly distributed throughout the CA1, CA2, and CA3 regions of the hippocampus [68]; confirming previous studies in rats [70,71]. The CA areas of the hippocampus are integral for learning and memory and are involved in AD pathology; an illness characterized by neurodegeneration and progressive loss of memory and cognitive function [72]. A decrease in VDR mRNA levels has been detected in human Alzheimer CA1 (34%) and CA2 (31%) pyramidal cells, but not in the temporal cortex or cerebellum (unaffected areas), as compared to controls with Huntington disease [73]. A long-term prospective study involving 498 elderly women demonstrated that women who did not develop AD after 7 year follow-up consumed 17% more dietary vitamin D at baseline versus women who developed AD [74]. However, there was no such difference between nonafflicted women and those who developed other dementias. Furthermore, women in the highest quintile for vitamin D intake at baseline decreased their risk (OR = 0.23) for developing AD versus the lowest quintile at the 7 year follow-up [74]. Similarly, using a group of 858 Italian adults aged 65 years+, the risk for cognitive decline at 6 year follow-up increased (OR = 1.60) with vitamin D deficiency (<25 nM) versus those who were sufficient (>75 nM) [75].

Parkinson's disease is a common neurodegenerative disease whereby selective death of dopaminergic neurons results in dysfunction characterized by tremors, impaired speech, and general loss of muscle control [76]. ALS and PD express some of the same pathophysiology. In PD, as in ALS, neuroinflammation presents as a prominent pathologic feature, characterized by activated microglia and infiltrating T cells at sites of spinal cord motor neuron injury [10,77]. ALS induces activation of microglia and increases their release of proinflammatory cytokines and free radicals such as TNF-α, IL-1β, inducible nitric oxide synthase, and O_2^- [78]. A

similar event occurs in PD patients with mutations in α-synuclein, with mutant protein aggregates causing activated-microglial release of a similar array of damaging biochemical factors [10,11]. There is also evidence to suggest that damage mediated by H_2O_2 and $^{\cdot}OH$ through the nonenzymatic Fenton reaction also occurs in PD [79,80]. Furthermore, as in ALS [81–83], PD electron transport chain (ETC) complex I activity is reduced, namely, in the substantia nigra of the brain [84–86]. Indeed, humans exposed to 1-methyl 4-phenyl 1,2,3,6 tetrahydropyridine develop PD through mechanisms that damage nigrostriatal ETC complex I [87].

Clinical vitamin D studies in human PD patients are scarce, but a 1997 case study [88] treated a hospitalized 50 year-old man diagnosed with PD with 4000 IU D_3/day and 1 g Ca/day (body weight not specified) in addition to regular therapy which alone failed to show any clinical benefits after 3 year. The patient exhibited low serum calcium, phosphorus, and 25(OH)D_3 prior to supplementation with D_3 + Ca. The patient improved significantly in the following year as evidenced by decreased rigidity and akinesia, with a substantive decrease in his multidrug therapy to only 375 mg levodopa/day. At 1-year follow-up, examination revealed absent tremor with only moderate rigidity.

The association of PD with low vitamin D status has been suggested through epidemiologic studies showing a higher prevalence of PD among those living in the more northern latitudes [89–92].

Vitamin D insufficiency (serum 25(OH)D_3 ≤75 nM) has also been observed in PD patients. PD patients have a significantly higher prevalence (55%) of hypovitaminosis D versus healthy controls (36%) and AD patients (41%) [93]. A high prevalence of low serum 25(OH)D_3 (<50 nM) in the mid-late summer months was found in patients with severe PD when compared with those with less advanced PD [94]. Sato et al. ascertained that PD patients also had lower serum 25(OH)D_3 (29.7 nM) as compared with healthy control subjects (83.2 nM). These researchers also found a significant and very strong inverse relationship between vitamin D status and the Unified PD Rating Scale III (r = –0.91) [95], a scale used to measure progression and severity of illness [96]. In a longitudinal study [97], a dose–response relationship was found between vitamin D status and risk for developing PD: those with a concentration of at least 50 nM had a relative risk one-third (RR = 0.33) of those with <25 nM. Furthermore, genetic VDR polymorphisms are associated with PD risk and age-at-disease-onset [98].

Multiple sclerosis is a demyelinating, neurodegenertative disease of the central nervous system. An association with higher latitude and susceptibility to developing MS is well established, as is the association of a higher later-in-life incidence of MS when born in late spring versus a lower incidence when born in late autumn [99]. In a prospective study involving American nurses, those who supplemented with at least 400 IU D_3/day were at a lower risk for MS versus those with no supplemental inake (RR = 0.59) [100].

In Vivo and *In Vitro* Animal Vitamin D Studies Related to ALS

Inflammation and Oxidative Stress

Human monocytes exposed to LPS exhibited a dose-dependent decrease in inflammation as measured by p38 phosphorylation in response to the administration of 15, 30, and 50 ng/mL 25(OH)D_3 [101]. Aged rats (20 months) supplemented with 1.05 μg 1,25$(OH)_2D_3$/kg b.wt./day for 21 days exhibited 25% lower IL-1β and 23% greater IL-10 expression versus nonsupplemented controls [102]. This was observed in tandem with 22% less amyloid- β oligomerization and 15% greater neprilysin (amyloid- β degrading enzyme) expression. In rodents, injection of type-2 collagen generates the collagen-induced arthritis (CIA) model; a model for RA [14]. CIA can be prevented by ingestion of 1,25$(OH)_2D_3$ in both mice and rats [16,17]. Alternatively, 1,25$(OH)_2D_3$ can prevent CIA from progressing from early to more severe stages [14]. VDR-deficient mice cross-bred with human TNF-α transgenic mice displayed signs of exacerbated degenerative arthritic disease including accelerated grip strength loss (47%), paw swelling (91%), and synovial bone erosion (106%) versus transgenic mice not deficient in VDR. When compared with wild-type mice, VDR-deficient mice exhibited an approximately 20 fold elevated serum level of TNF-α [103], underscoring the role of VDR ligands in modulating inflammation.

Under normal circumstances, inducible nitric oxide synthase is not expressed by glia, however in diseases involving the CNS such as MS [104], AD [105] and PD [106] its expression in glia is an inherent aspect of pathology. In ALS, nitric oxide production is involved in the conversion of O_2^- to $ONOO^-$, a process that occurs at a rate 3× faster than the rate at which normal SOD can catalyze the dismutation of the O_2^- radical to H_2O_2 [107]. This event leads to protein nitration and damage to the cytoskeletal structure and enzymes [108] and can contribute to cell death [109]. Indeed, nitric oxide synthase levels are found to be elevated in the G93A mouse spinal cord versus control, an event that parallels gliosis and motor neuron loss [110]. Since vitamin D has been shown to inhibit nitric oxide synthase in rodents [111,112], a similar inhibition in ALS animal models or human patients could help mitigate disease pathology.

Rodent experimental allergic encephalomyelitis (EAE, used as a model of human MS) shares common pathophysiology with ALS. Similar to motor neuron destruction in ALS, oligodendrocytes (the myelin-producing cells of the CNS) are vulnerable to glutamate excitotoxicity [113]. Treatment with glutamate receptor antagonists in this model increased oligodendrocyte survival and decreased markers for axonal degeneration [114,115] which correlated with improved EAE rodent disease score [115]. Furthermore, glutamate antagonists in this model reduced ventral horn motor neuron loss; neurons of central importance in ALS pathology [114]. It follows that Riluzole (the only established and moderately effective drug-based treatment for ALS) is also effective in reducing inflammation, demyelenation, axonal damage, and overall disease severity in rodent EAE [110]. Vitamin D treatment may follow a similar mechanism, since *in vitro* administration also protected rodent cortical neurons against glutamate excitotoxicity [117,118].

In vivo, dietary administration of 20 ng 1,25$(OH)_2D_3$ 1 day before EAE disease induction (35–56 days of age) fully prevented onset of disease, whereas all control mice fed regular chow became paralyzed in both fore and hind limbs. In the same study, a 300 ng intraparietal injection of 1,25$(OH)_2D_3$ at the first sign of symptoms (limp tail) 10 days after myelin basic protein immunization (45–66 days of age) halted the advancement of the disease

for the remainder of the observation period (approximately 30 days) [13], whereas the controls developed paralysis in both fore and hind limbs. To test if the protective effect is reversible, $1,25(OH)_2D_3$ was removed from half of the vitamin D-treated mice at age 63–84 days, thus creating three different groups: (1) mice maintained on $1,25(OH)_2D_3$ throughout the study, (2) mice temporarily provided with, then restricted of, $1,25(OH)_2D_3$, and (3) mice fed a diet devoid of $1,25(OH)_2D_3$ throughout the study. Group 3 experienced more severe signs of disease as compared to the other two groups, however, group 2 eventually caught up with group 3 in disease severity 10 days post-$1,25(OH)_2D_3$ withdrawal. This strongly establishes that $1,25(OH)_2D_3$ supplementation interferes with EAE.

Vitamin D also has direct antioxidant effects *in vivo*. Injection of 0.6 pmol $1,25(OH)_2D_3$ into rat substantia nigra reduced zinc-induced lipid peroxidation and dopamine loss by approximately 20% and 33%, respectively, after 7 days versus zinc alone [6]. As well, $1,25(OH)_2D_3$ reduced zinc-induced substantia nigra apoptosis as evidenced by significantly reduced presence of cytosolic cytochrome *C*. $1,25(OH)_2D_3$ pretreatment for 15 days (i.p. 5 IU/g b.wt./day) in diabetic rats increased the enzyme activity of liver and kidney catalase, glutathione peroxidase, and SOD1 by approximately 2–4.4 fold, while simultaneously decreasing lipid peroxidation as indicated by thiobarbituric acid reactive substances by 40–46% versus controls, indicating a reduction in oxidative stress-induced damage [119].

Muscle

G93A mice transgenically overexpress the mutant human SOD1 gene and follow the same disease pattern as human ALS clinically and neuropathologically [120,121], and is thus the most widely used animal model of ALS. We have previously demonstrated that dietary D_3 supplementation at 10-fold (10 IU D_3/g feed) the adequate intake (AI, 1 IU D_3/g feed) delays the decline in paw grip endurance and motor performance by 7% and 22%, respectively, versus the AI in the transgenic G93A mouse model for ALS [21]. In a later blinded study, dietary D_3 at 50-fold the AI (50 IU D_3/g feed) delayed the decline in paw grip endurance by 12% versus the AI [22]. Alternatively, D_3 restriction (0.025 IU D_3/g feed) decreased cumulative scores for paw grip endurance and motor performance postdisease onset by 23% and 18%, respectively, versus control [23]. Complete analysis of the mouse skeletal muscle tissue to elucidate what molecular aspects are regulated by this vitamin D supplementation will be forthcoming [122–125]. Despite the observed improvements in functional ability in the two supplementation studies, there were no significant differences in age at disease onset, duration of disease progression or lifespan. However, it is of note that the AI mice (the control mice) were likely to be consuming D_3 at levels considerably above what is truly adequate [21,22].

In vitro, mouse skeletal muscle cells treated with 100 nM $1,25(OH)_2D_3$ exhibited increased expression and nuclear translocation of the VDR and decreased cell proliferation versus placebo. $1,25(OH)_2D_3$ treatment also promoted myogenic differentiation by increasing IGF-II and follistatin expression, while decreasing myostatin expression; the only known biological inhibitor of muscle mass [126]. In other studies, cultured skeletal and cardiac muscle cells demonstrate increased calcium uptake following exposure to physiological concentrations of $25(OH)D_3$ or $1,25(OH)_2D_3$ [127,128]. Additionally, $1,25(OH)_2D_3$ treatment at physiological concentrations elevated cell density and fusion in chick skeletal muscle cell culture, indicating a role for vitamin D in muscle cell proliferation and differentiation [129]. The improved functional capacity in G93A mice [21,22], as well as the improved musculoskeletal function and reduction in falls observed in human studies [54–58,60,61] following D_3 supplementation may also be due to muscle-specific mechanisms involving contractile protein synthesis and energy homeostasis. In D_3-deficient rats, a single oral dose of 400 IU D_3 significantly increased muscle leucine incorporation (a measure of muscle protein synthesis) at 7 h compared with untreated controls [130]. Intravenous injection of 0.4 μg $25(OH)D_3$ significantly increased intramuscular leucine concentrations at 4 h, whereas removal of the kidneys [and therefore the ability to renally convert $25(OH)D_3$ to $1,25(OH)_2D_3$] did not abolish this effect [130], suggesting a direct role of $25(OH)D_3$ independent of $1,25(OH)_2D_3$ in muscle function. In evidence, rat epitrochlear muscle had greater leucine incorporation and ATP content in a medium containing 50 nM $25(OH)D_3$ versus untreated muscle. Administration of D_3 at 52,000 nM had no measurable effect, indicating that D_3's action in skeletal muscle is conditional upon its conversion to $25(OH)D_3$, and that $25(OH)D_3$ is the active genomic vitamin D metabolite in skeletal muscle. The authors found no measureable effect of 1.25 nM $1,25(OH)_2D_3$ on muscle amino acid incorporation or ATP content versus untreated muscle [130]. In support, hatchling chicks fed a D_3-free diet for 2 week followed by 1 week supplementation with 80 IU/day D_3 had greater concentrations of the contractile proteins actin and troponin C compared with chicks maintained on the D_3-free diet [131].

Neurodegeneration and Neuroprotection

In vivo, radiolabelled $1,25(OH)_2D_3$ uptake in mouse spinal cord 3–4 h postinjection (1 or 3.8 ng/g b.wt.) was clearly strongest in the nuclei of large motor neurons of the spinal cord anterior horn, even in animals that received the lower dose [69]. This demonstrates the presence of the nuclear VDR particularly in the large motor neurons of the spinal cord, indicating a role for vitamin D in maintaining the health of motor neurons; cells which are destroyed in ALS.

Vitamin D receptor-knockout mice have significant locomotor and muscular functional impairment but no apparent cognitive dysfunction, in line with human ALS characteristics [132]. Recently, in the cuprizone mouse model of MS, high doses of dietary D_3 (6.2 and 12.5 IU/g feed) significantly attenuated brain white matter demyelination and microglia activation [133]. Rats orally administered 500 IU D_3/kg b.wt./day for 12 weeks after surgical peroneal nerve injury exhibited a 71% greater number of axons in the proximal area of injury versus vehicle-only treated rats. Furthermore, D_3-supplemented rats demonstrated 8% greater proximal and 10% greater distal myelination; assessed using the G-ratio (defined as the ratio of axon diameter to myelinated fibre outer diameter) [134]. Animal cerebral artery ligation involves the over-release of excitatory amino acids, overinflux of calcium into the cell, oxidative stress, mitochondrial respiratory

damage, and programmed cell death [18]; pathologic mechanisms shared by ALS. Rats pretreated with $1,25(OH)_2D_3$ for 8 days (i.p., 1 ng/g b.wt./day), but not 4 days, exhibited 2.3-fold less volume of infarcted brain tissue due to 90 min cerebral artery ligation versus controls [18]. This protection can be at least partially explained by the nearly 2-fold increase in glial-derived neurotrophic factor (GDNF) endogenous protein expression; a finding confirmed by what has previously been demonstrated *in vitro* [135]. The same group showed that rats lesioned with 6-hydroxydopamine after being pretreated for 8 days with $1,25(OH)_2D_3$ (1 ng/g b.wt./day) had hypokinesia significantly attenuated 1 month postlesioning. This was evidenced by approximately 35–100% greater locomotor activity versus saline-treated rats [12]. *In vitro* work showed that $1,25(OH)_2D_3$ pretreatment attenuated H_2O_2-induced neuronal cell death by approximately 3.4-fold versus saline pretreatment. Even in healthy wild-type rats, $1,25(OH)_2D_3$ administration (i.p., 1 ng/g b.wt./day) for 7 days increased brain GDNF protein expression by 40% versus saline controls [19]. Various other studies have demonstrated that $1,25(OH)_2D_3$ acts on cells of the nervous system *in vitro* to increase synthesis of other neurotrophic factors which promote neuronal survival, growth, development, and maintenance such as, neural growth factor [136–138], and neurotrophin-3 [139].

Vitamin D may also exert neuroprotective effects through the upregulation of calcium-binding proteins. Specific groups of motor neurons such as those found in Onuf's nucleus and the oculomotor nerve are resistant to the ALS degeneration observed in other neurons [140]. Motor neurons of Onuf's nucleus and the oculomotor nerve are responsible for the bladder/rectal functions and eye movement often preserved in ALS, even in the late stages of the disease [141,142]. Protection in these neurons may be attributed to the greater expression of calbindin and/or parvalbumin versus neurons which are lost early in ALS [142–145]. Spinal cord analysis in G93A mice showed that parvalbumin-positive anterior horn motor neurons were severely diminished versus controls before the onset of symptoms, whereas calbindin-positive neurons were mostly preserved [146]. During the symptomatic stage, however, parvalbumin and calbindin immunoreactivity was almost completely abolished. In the brain stem, oculomotor and abducens motor neurons which stained parvalbumin-positive were as well preserved in transgenic mice as in the controls, even at the end-stage of disease [146]. Indeed, G93A mice with enhanced levels of parvalbumin experienced delayed disease onset by 17% and extended survival by 11% versus controls, accompanied by a 33% greater rate of lumbar spinal cord neuronal survival [147].

Vitamin D increases expression of calcium-binding protein *in vivo*. Rats fed 20 IU D_3/g b.wt./day for 113 days via gastric cannulation exhibited a 50% increased basal ganglia parvalbumin protein expression versus controls (approximately 0.15 IU D_3/g b.wt./day), although no significant changes were found in total cortex or total hippocampus [20]. These changes occurred despite multiple signs of D_3 toxicity in the animals receiving the extremely high dose of D_3 [20]. Separately, rats intracerebroventricularly injected with $1,25(OH)_2D_3$ (80–250 ng) exhibited strong parvalbumin, calbindin, and calretinin protein immunoreactivity in spinal cord motor neurons versus control, with the strongest detection occurring with 100 ng [148].

In addition to the potential for vitamin D in mitigating the sequelae of hyper-intracellular calcium concentrations in motor neurons in ALS, vitamin D could also mitigate the severity of ALS by attenuating glutamate excitotoxicity-induced motor neuron death. Chronic $1,25(OH)_2D_3$ treatment of rat cortical neurons provided cellular protection against glutamate excitotoxicity in a dose-dependent fashion, where 10 and 100 nM $1,25(OH)_2D_3$ allowed for 10% and 30% more neuronal survival, respectively, versus control [117]. Furthermore, cells treated with $1,25(OH)_2D_3$ increased the expression of the neuronal markers microtubule associated protein-2, growth-associated protein-43, and synapsin-1, suggesting a neuroprotective role for $1,25(OH)_2D_3$ [117]. Separately, $1,25(OH)_2D_3$ at 100 nM protected mouse neocortical and hippocampal neurons from glutamate insult versus controls despite a delay of 6 h after the initiation of an excitotoxic challenge [118].

Limitations

Different diet-based interventions in rodent models of ALS have yielded varying effects on disease onset, lifespan, and/or functional capacity [149]. Unfortunately, successful interventions in rodent models have not translated well to human clinical trials due to poor design, lack of statistical power, as well as the fact that nearly all animal studies commence prior to disease onset whereas clinical trials are initiated at far more advanced stages of disease [149]. Thus, it remains to be seen if the beneficial effects of high dose vitamin D supplemtation observed in rodents [21,22] will translate to their human counterparts.

Future Directions

Future animal research should measure the effect of the D_3 supplementation on markers related to mechanisms implicated in ALS pathophysiology. As such, markers of oxidative stress, antioxidant capacity, inflammation, apoptosis, and neuron count should be measured in the brain/spinal cord/skeletal muscle. As well, the quantification of skeletal muscle contractile proteins would be useful to establish the mechanism for the observed improvement in muscle function and motor performance observed following vitamin D supplementation, as well as the decrement in funcational capacity observed following vitamin D restriction, in the high copy G93A transgenic mouse model of ALS [21–23]. Since vitamin D_3 toxicity was observed in G93A females [22], protein analysis should be conducted to confirm the absence of 1α-OHase in skeletal muscle as well as the corresponding $1,25(OH)_2D_3$ and $25(OH)D_3$ concentrations, which could explain the observed improvement in functional capacity despite overall toxicity. In addition, vitamin D status, calcium concentrations, and intracellular calcium trafficking/buffering capacity should be measured to establish levels at which toxicity is induced in females, to compare these values to those in males, as well as to understand the role of calcium binding proteins in the cytosol, endoplasmic reticulum, and mitochondria in modulating the sequelae of ALS. Also, of importance is the establishment of a dose which is closer to optimal concerning its effects on functional capacity in this model. This should be done using differential doses of D_3. Since 50 IU/g feed is likely to be the approximate threshold for D_3 toxicity, we

would recommend future experimental high vitamin D doses to be lower than this amount. It would also be of value to experimentally explore the effect of $1,25(OH)_2D_3$ and/or noncalcemic vitamin D analogs in this disease model. Most importantly, studies should be conducted to confirm high dose vitamin D safety in ALS patients, as well as to test the efficacy, if any, of vitamin D on the rapid progression of human ALS pathology.

Conclusion

Vitamin D exerts its influence on a wide variety of different physiological processes in both the healthy and diseased states. Vitamin D may be used as an effective therapy in ALS based on the evidence regarding its effect on muscle function, oxidative stress, inflammation, neuroprotection, mitochondrial function, and apoptosis, *in vivo* in humans and rodents, as well as *in vitro*. In addition, vitamin D's influence on diseases which share pathophysiological similarities with ALS suggest that vitamin D may also attenuate ALS pathology. This hypothesis warrants testing in randomized, blinded clinical trials. We have previously shown that vitamin D_3 at 10 and 50 IU/g feed (approximately 1.7–8.1 IU D_3/g b.wt./day) in the G93A mouse attenuated the decline in functional capacity. Furthermore, we have also shown that vitamin D_3 restriction (0.004 IU D_3/g b.wt./day) worsened functional capacity in the same mouse model. Since it has been shown to effect multiple aspects of ALS pathophysiology, vitamin D is a strong candidate as a therapeutic for ALS.

Conflict of Interest

The authors declare no conflict of interest.

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