Explore the Neuroprotective Role of Vitamin D3 in 3-Nitropropionic Acid Induced Mouse Model of Huntington's Disease

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By

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CERTIFICATE

This is to certify that the thesis entitled “Explore the neuroprotective role of Vitamin D3 in 3-nitropropionic acid induced mouse model of Huntington’s disease” and submitted by SK VENKATAMANJARI, ID No. 2018PHXF0002H for the award of the Ph.D. degree of the institute embodies original work by her under my supervision.

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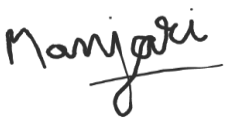
Associate Professor
Date: 09-02-2024

Declaration

I hereby declare that the work which is presented in the thesis, entitled "Explore the neuroprotective role of Vitamin D3 in 3-nitropropionic acid induced mouse model of Huntington's disease" in the fulfilment of requirements for the award of the degree of Doctor in Philosophy from the department of Biological Sciences and submitted to Birla Institute of Technology and Science, Pilani is an original piece of research work under the guidance of Dr. Pragya Komal, Birla Institute of Technology and Science, Pilani, India. The matter embodied in this thesis has not been submitted by me for the award of any other degree of any other Univeristy/ Institute.



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Abstract

Vitamin D3 (cholecalciferol; VD) is an essential micronutrient, extensively agreed as the first-line medication for bone-related disorders due to its involvement in calcium/phosphorus level maintenance. Recent research highlights also shed its importance in several brain-related disorders where it regulates redox imbalance, inflammation, apoptosis, growth-factor synthesis, synaptic plasticity, and neurotransmission. VD deficiency is proposed to be a risk factor in the progression of several neurological disorders, including Huntington's disease (HD). The majority of the biological activity is mediated by the active metabolite of VD i.e., 1,25-hydroxyVitamin D3 [1,25$(OH)_2$D3 (calcitriol)] via binding to Vitamin D receptor (VDR). VD-VDR interaction in turn regulates several genes involved in neuroplasticity, neuroprotection, neurotropism, and neuroinflammation. The majority of the work reflecting the neuroprotective function of VD comes from clinical and preclinical studies conducted in neurological diseases like Alzheimer's disease (AD), and Parkinson's disease (PD), where reports in the field of Huntington's disease (HD) remain limited. In this thesis, I have explored the neuronal benefits of VD supplementation using a toxic-induced model of HD, 3-nitropropionic acid (3-NP) that mimics some of the neuropathological symptoms observed in HD.

My study is broadly divided into two parts where in the first part I have assessed the phenotypic effects of two doses (500IU/kg/day and 2000IU/kg/day) of VD supplementation on movement, motor coordination and memory function in 3-NP induced mouse model of HD. The results drawn from the first part of the study inferred that 500IU/kg/day VD dose was sufficient to rescue movement impairment observed in HD mice. Hence for the latter part of my thesis I restricted my studies on only 500IU dose of VD.

The second part of my thesis showed that the benefits of VD supplementation on behavior phenotype occurred via an enhancenemnt in the gene expression of neurotrophins like brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). VD induction not only enhanced neurotrophin levels but also increased cholinergic neurotransmission in two vital regions of the brain severely affected in HD i.e the striatum and the cortex. I showed that VD-VDR interaction restored the

cholinergic signaling and increased the protein expression of alpha 7 nicotinic acetylcholine receptors (α7nAChRs). An enhancement in the gene/protein expression of α7nAChRs occurred with a concomitant reduction in the gene expression of brain resident immune protein, the T-cell receptor beta (TCR-β) subunit in HD mice. Overall, in the present thesis I showed an anti-oxidant, anti-apoptotic, anti-cholinesterase and anti-inflammatory effects of VD in HD.

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List of Abbreviations

1,25-MARRS: 1,25-membrane-associated rapid response steroid-binding proteins
3-NP: 3-Nitropropionic acid
6-OHDA: 6-hydroxydopamine
ACh: Acetylcholine
AChE: Acetylcholinesterase
AD: Alzheimer's disease
AF-2: Activation domain
Akt: Serine/threonine kinases
ALS: Amyloid lateral sclerosis
ARE: Antioxidant response element
Bax: Bcl-2 associated X protein
BBB: Blood-brain barrier
BCl-2: B-cell lymphoma 2
BDNF: Brain derived neurotrophic factor
BSA: Bovine serum albumin
CamKII: calmodulin-dependent protein kinase II
Cat: Catalase
CCL2: Chemokine (c-c motif) ligand 2
CD163: Cluster of differentiation 163
CD204: Cluster of differentiation 204
CD206: Cluster of differentiation 206
CD3ζ: A protein complexed receptors for MHC-I
CD86: Cluster of differentiation 6
CHDRDL1: Chordin like 1
CNS: Central nervous system
CREB: Cyclic-AMP response element binding protein
c-Ret: c-terminal of rearranged during transfection
CYPs: Cytochrome P450 oxidases
DA: Dopamine
DBD: DNA-binding domain
DBP: Vitamin D binding protein
DR3: Spacer DNA
DVD: Developmental Vitamin D3 deficiency
EAE: Experimental autoimmune encephalomyelitis
ERK1/2: Extracellular signal-related protein kinase 1 and 2
ERp57: Endoplasmic reticulum stress protein 57
GABA: Gamma-aminobutyric acid
GDNF: Glial derived neurotrophic factor
GnRH: Gonadotropin-releasing hormone
GP: Globus pallidus
GPe: Globus palladius externa
GPi: Globus palladius interna

GpX: Glutathione peroxidase
GSH: Glutathione
HATs: Histone acetyltransferases
HD: Huntington's disease
HDACs: Histone deacetylases
HEAT: Huntingtin, elongation factor 3, protein phosphatase 2A, and TOR1 [target of rapamycin 1]
Iba-1: Ionized-calcium binding adapter protein-1
IFN-β: Interferon-beta
IFN-γ: Interferon-gamma
IKKB: I-kappaB kinase
IL-10: Interleukin-10
IL-1β: Interleuikin-1 beta
IL-4: Interleukin-4
IL-6: Interleukin-6
KLF4: Kruppel like factor 4
LBD: Ligand-binding domain
LNCaP: Lymph node cancer of the prostate
LTP: Long-term potentiation
L-VGCC: L-type voltage-gated calcium channels
mAChRs: Muscarinic acetylcholine receptors
MAPK: Mitogen activated protein kinase
MBP: Myelin basic protein
MDA: Malondialdehyde
MHC-I: Major histocompatibility complex-I
MHC-II: Major histocompatibility complex II
mRNA: Messenger ribonucleic acid
MS: Multiple sclerosis
MSNs: Medium spiny neurons
mVDR: Membrane bound Vitamin D receptor
MWM: Morris water-maze test
nAChRs: Nicotinic acetylcholine receptors
NcoRT2: Nuclear receptor corepressor 2
NF-κB: Nuclear factor kappa B
NGF: Nerve growth factor
NMDA: N-methyl-D-aspartate
NOS: Nitric oxide synthase
NOX: Nitrogen oxide
Nrf2: Nuclear factor erythroid 2 related factors
NT-3: Neurotrophin-3
NT-4: Neurotrophin-4
nVDR: Nuclear Vitamin D receptor
PCNA: Proliferating cell nuclear antigen
PD: Parkinson's disease

PDIA3: Protein disulfide isomerase 3
PGC-1α: Peroxisome proliferator-activated receptor-gamma coactivator
PI3K: Phosphoinositol-3-kinase
PKA: Protein kinase A
PLA2: Phospholipase A2
PLC: Phospholipase C
PSD95: Post-synaptic density protein 95 (PSD95)
PVDF: Polyvinylidene fluoride
ROS: Reactive oxygen species
RunX: Runt-related transcription factor
RXR: Retenoid X receptor
SCZ: Schizophrenia
SDH: Succinate dehydrogenase
siRNA: Small interfering RNA
Sirt1: Sirtuin 1
SMRT: Silencing mediator of retinoic acid and thyroid hormone receptor
SN: Substantia nigra
SNAP25: Synaptosomal associates protein 25
SNc: Substantia nigra pars compacta
SNr: Substantia nigra pars reticulata
SOD: Superoxide dismutase
Sp: Substance p
SRC1: Steroid receptor coactivator 1
Stat1: Signal transducers and activators of transcription
TAC: Total antioxidant capacity
TASK-1: TWIK-related acid sensitive potassium channel 1
TBZ: Tetrabenazine
TCR: T-cell receptor
TGF-β1: Transforming growth factor-beta subtype 1
TH: Tyrosine hydroxylase
TLR-10: Toll-like receptor 10
TLR-4: Toll-like receptor 4
TNFPAIP6: Tumor necrosis factor-alpha-induced protein 6
TNFS4: Tumor necrosis factor superfamily number 4
TNF-α: Tumor necrosis factor-alpha
TrkA: Tyrosine receptor kinase A
TrkB: Tyrosine receptor kinase B
TrkC: Tyrosine receptor kinase C
TRP: Transient receptor potential
TRPV1: Transient receptor potential vanilloid family 1
TRPV6: Transient receptor potential vanilloid family 6
TWIK: Tandem of p domains in a weak inward rectifying potassium channel
VCAM: Vascular cell adhesion molecule 1
VD: Vitamin D3

VDR: Vitamin D receptor
VDRE: Vitamin D response element
α7 nAChRs: α7 nicotinic acetylcholine receptors (CHRNA7)
α-BTX: α-Bungarotoxin

Chapter 1

Introduction

1.1. Overview and rationale:

In the year 1922, McCollum and colleagues discovered Vitamin D for the treatment of rickets (McCollum et al., 1922). Two years later, Vitamin D was named according to the source of origin and difference in the chemical structure (Hess and Weinstock, 1924). In plants, it is considered Vitamin D2 (ergocalciferol), and from animal sources, it is referred to as Vitamin D3 (VD, cholecalciferol; Hess and Weinstock, 1924). In 1936, a study by Windans and Bock discovered that when skin gets exposed to ultraviolet (UV) rays (290-315nm) 7-dehydrocholesterol was converted into pre-Vitamin D3 which further gets isomerized to form Vitamin D3 (VD) or cholecalciferol (Windans and Bock, 1936). Thus, VD was obtained through dietery sources and oral supplements. Multiple studies thereafter reflected the importance of VD in the maintenance of bone homeostasis (Carpenter and Zhao, 1999; Holick et al., 1977; Zhang and Naughton, 2010). The physiological benefits of VD were also observed in diseases like osteoporosis, hyperthyroidism, cardiovascular diseases, diabetes, hypertension and cancer (Alvarez et al., 2019; Bouillon et al., 2022; Hou et al., 2018; Geleijnse, 2011; Laird et al., 2010; Nakashima et al., 2016; Pilz et al., 2016; Varghese et al., 2021; Vaughan-shaw et al., 2017; Walker and Bilezikian, 2017; Wanger et al., 2012).

In the last three decades, numerous studies showed therapeutic benefits of VD on brain health (da Costa et al., 2023; Eyles et al., 2005, 2013; Emmanuel Garcion et al., 2002; Gezen-Ak et al., 2007; Grimm et al., 2013; Koduah et al., 2017; Landfield and Cadwallader–Neal, 1998; Lemire and Archer, 1991; Musiol and Feldman, 1997; Patel and Shah, 2022; Stio et al., 1993). The therapeutic options of VD on brain health largely come from observational and clinical studies performed on human samples and animal models where it was demonstrated that VD deficiency increases the risk of mortality, infections, and many other diseases which possibly can be avoided by VD supplementation (Amrein et al., 2020; Harrison et al., 2020; Janjusevic et al., 2022; Siddiqui et al., 2020; Smolders et al., 2021; Wajda et al., 2019). VD deficiency is now considered a global pandemic (Anjum et al., 2023). The prevalence of health problems associated with low serum levels of VD has been observed among more than a billion people worldwide (Moretti et al., 2018). Several scientific groups observed that the variations in the amount of VD in the

body are attributed to geographical location, seasons, cultural practices, lifestyles, and diets (Webb, 2006). Several factors determine the body's availability of VD, but 90% of the elderly people are deficient in VD because of their lifestyle and low exposure to sunlight (Rui et al., 2014). In 2017, Michael Berridge proposed aging as an important risk factor for the development of neurodegenerative disorders (Berridge, 2017). In his study, he hypothesized that the process of aging could be modulated by adequate intake of VD supplementation that in turn regulated the expression of genes related to calcium homeostasis, antioxidants, serotonin synthesis, inflammation, autophagy, mitochondrial dysfunction, epigenetic changes and DNA disorders (Berridge, 2017). He quoted VD as a "miracle Vitamin" as VD sufficient individuals were observed to have a decreased rate of aging (Berridge, 2017). This has led to considerable debate regarding VD supplementation in the elderly and whether deficiencies in VD represent an indicator of ill health or increase one's susceptibility to chronic disease (Berridge, 2017).

VD deficiency and insufficiency were observed across myriad age-related neurological diseases, such as Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS), Huntington's disease (HD), dementia and depression (Anwar et al., 2023; Azam et al., 2021; Banerjee and Chatterjee, 2003; Berridge, 2017; Chel et al., 2013; Fullard and Duda, 2020; Moretti et al., 2018; Munger et al., 2004; Somoza-Moncada et al., 2023). One of the major significant mental health problems today observed in elderly people includes dementia and impairment in movement which has been shown with an inverse relationship with circulating VD levels in the blood plasma as well as in the brain (Bivona et al., 2019; Farghali et al., 2020; Sultan et al., 2020; Utkan Karasu and Kaymak Karataş, 2021). A high concentration of VD metabolites in the brain regions particularly the hippocampus and substantia nigra region has been shown to improve memory and motor function (Moretti et al., 2018). In the other brain regions like the prefrontal cortex, vital for attention and working memory, low levels of VD have been positively correlated with anxiety induction (Wu et al., 2021). Molecular studies have suggested that aging and age-related neurological disorders share some common characteristics such as protein aggregation, synaptic dysfunction, energy depletion from mitochondrial abnormalities, DNA and RNA deficits, inflammation, oxidative stress, and neuronal death (Wilson et al., 2023). The patients suffering from these neurological conditions showed a dramatic

improvement in cognitive function, learning, and memory on VD supplementation (Eyles, 2020; Gil and Rego, 2008; Karabulut et al., 2021; Landel et al., 2016; Liang et al., 2018; Lima et al., 2018; Mohamed et al., 2015; Rossom et al., 2012; Yamini et al., 2018). Thus, multiple studies showcased the neurobiological benefits of VD intake in neurodegenerative diseases.

Huntington's disease (HD) is one such neurodegenerative disorder where a high prevalence of VD deficiency was reported in the Caucasian population (Chel et al., 2013). However, in the case of HD, minimal data is available to determine the molecular mechanism behind the benefits of VD supplementation and its mode of action in the striatum, the prime brain region known to undergo neuronal atrophy in HD. The striatum constitutes the main output station of the basal ganglia circuitry and the medium spiny neurons (MSNs) are the major neuronal components of the striatum (Chambon et al., 2023). These neurons are demarcated on the basis of the expression of dopamine (D1 and D2) receptors and are involved in motor control and cognitive functions. D2 receptors are expressed in indirect pathway neurons and inhibit motor output, while D1 receptors are expressed in direct pathway neurons and have an excitatory effect on motor output. In order to control movement appropriately, the balance between direct and indirect pathways is essential. HD is one such movement disorder caused by alterations in the direct and indirect pathways (Garret et al., 2018; Manjari et al., 2022; Chambon et al., 2023). It is a neurodegenerative disorder caused by a selective loss of medium spiny neurons (MSNs) primarily in the striatum (Gil and Rego, 2008; Gil-Mohapel, 2012). HD is an autosomal dominant disorder and is caused by an increased number of CAG repeats in the 1^{st} exon of the huntingtin gene *(Htt)*, located on the 4^{th} chromosome (McColgan and Tabrizi, 2018; Capiluppi et al., 2020). The expansion of more than 36 CAG repeats causes a polyglutamate stretch in the huntingtin protein where the accumulation of polyglutamate aggregates causes loss of striatal neurons. Some of the features which are responsible for this selective neuronal loss include mitochondrial dysfunction, oxidative stress, inflammatory responses, unbalanced homeostasis of apoptotic molecules, impairment in synaptic plasticity, reduced axonal transport of neurotrophins and neurotransmitters (Cattaneo et al., 2005; El-Sahar et al., 2020; Garret et al., 2018; Gatto and Weissmann, 2022; Gil and Rego, 2008; Johri et al., 2013; Paul and Snyder, 2019; Ravalia et al., 2021;

Sawa et al., 2003; Upadhayay et al., 2023; Zheng et al., 2018). Therefore, in our study, I have mainly focused on exploring the potential mechanisms of neuroprotection by Vitamin D3 (VD) in Huntington's disease (HD). In my thesis, I have examined the phenotypic changes in the behavior of HD animals on VD supplementation. My work also unravels the molecular underpinnings by which VD shows its protective role in the striatum via the Vitamin D receptor (VDR) in 3-nitropropionic acid (3-NP) induced mouse model of HD.

1.2. Existing Research Gaps:

1. The beneficial effect of VD supplementation in HD patients remains limited.
2. There is limited data available in which striatal brain samples from HD model have been examined for action of VD supplementation on VDR expression.
3. No study has yet validated VD potential to activate multiple rescue pathways and decrease the progression of striatal neuron death in HD.
4. There is limited information about neuroprotective role of VDR signaling pathways and its downstream cellular targets in HD.

1.3. Research Objectives and Hypothesis:

1.3.1. Research objectives:

Evidence indicates that VD supplementation has a beneficial effect on cognitive and psychiatric functions by regulating the expression of key neurotrophins like brain derived neurotrophic factor (BDNF), nerve growth factor (NGF), glial derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4) (Alamro et al., 2020; Koshkina et al., 2019; Lin et al., 2020; Mohamed et al., 2015). The protective effect of VD is commonly carried out through its biological receptor i.e. VDR (Bao et al., 2020; Lima et al., 2018; Lin et al., 2020; Xu and Liang, 2021). One pathway which is known to increase the neuronal survival by the cross-talk between VDR and cholinergic activity via alpha 7 nicotinic acetylcholine receptor (da Silva Teixeira et al., 2020; Zaulkffali et al., 2019).

Thus, in the first part of my study, I examined the beneficial effects of VD supplementation on behavior, thereby rescuing the phenotypic changes in HD. In the second part of my study, I explored the molecular mechanisms through which VD

supplementation showed its rescue effect, which helps in the survival of striatal neurons. In the third part of my thesis, I have determined VD's anti-inflammatory, antioxidant, and anti-cholinesterase activity on HD in cortical and striatal neurons. In the final part of my study, I have discussed the importance of a nutraceutical, VD as a savior for network-wide function through the cross-talk between the VDR and the cholinergic activity through alpha 7 nicotinic acetylcholine receptor. In the future direction, I have argued on the starling role of VD, where its early intake can benefit in aging and across age-related neurological disorders including HD.

1.3.2. Aim and objectives:

The main aim of my study was to explore the benefits of VD in HD and elucidate the downstream signalling mechanisms of neuroprotection. The following were the proposed objectives of the present thesis:

1. To understand the dose and time-dependent effect of VD supplementation in the 3-nitropropionic acid (3-NP) induced mouse model of HD.
2. To explore if VD enhances neurotrophin expression via VDR in HD mice.
3. To elucidate the anti-oxidative, anti-inflammatory, and anti-cholinesterase activity of VD in HD.
4. To explore VD-mediated intervention on α7 nAChRs and TCR-β subunit receptor gene expression in HD.

1.4. Background:

1.4.1. The historical significance of VD:

In 1921, Hess & Unger discovered from their clinical studies that sunlight could cure rickets (Hess and Unger, 1921). Later, in the year 1922, McCollum and his coworkers discovered Vitamin D, while trying to discover a drug to treat rickets (McCollum et al., 1922). McCollum and his co-workers demonstrated that a modified form of cod-liver oil, which was depleted of Vitamin A, cured rickets. His team identified Vitamin D as an entity of fat-soluble Vitamins (McCollum et al., 1922). In successive years, a link between sunlight and calcium retention was provided by the other authors when they found that

rickets in rats got cured by irradiating food with UV rays (Steenbock, 1924). UV irradiation converts inactive lipids into active antirachitic substances which potentiated bone development in children (Steenbock, 1924). Later, Hess & Weinstock named animal-derived Vitamin D as Vitamin D3 (VD), while plant-derived Vitamin D was tagged as Vitamin D2 (Hess and Weinstock, 1924). In 1936, the dermal synthesis of Vitamin D3 via sun irradiation was explored by Windans and Bock, who showed non-enzymatic production of Vitamin D3 from 7-dehydrocholesterol in the skin on exposure to sunlight (Windans and Bock, 1936). In 1960s, the structural details of VD revealed that it was a steroid hormone which can act as a nuclear receptor (Azam et al., 1969; Colston et al., 1981; Haussler et al., 1995, 1968; Norman et al., 1982).

The effect of VD on bone health remained undebatable but the first evidence on the neurobiological importance of VD was provided by Lemire and Archer in the year 1991 (Lemire and Archer, 1991). Their study reflected calcitriol as an important remyelinating and immunosuppressive agent, where in an animal model for multiple sclerosis (MS) i.e., experimental autoimmune encephalomyelitis (EAE), VD supplementation increased the expression of myelin basic protein (MBP) in the serum (Lemire and Archer, 1991). Since 1991, researchers discovered progressively the myriad benefits of calcitriol intake across a number of neuropsychiatric disorders where studies highlighted the role of calcitriol in neuroprotection, immunomodulation, neurotransmitter maintenance, and synaptic plasticity (Almeras et al., 2007; Cornet et al., 1998; Eyles et al., 2007, 2005; Garcion et al., 1997; Hajiluian et al., 2017; Lv et al., 2020; Rodrigues et al., 2019; Shinpo et al., 2000). Subsequent studies showed VD mediated regulation on synaptic neurotransmission and synaptic plasticity via enhancement in multiple downstream signal transduction pathways (DeLuca et al., 2013; Eyles et al., 2007; Groves et al., 2014; Kouba et al., 2023; Mayne and Burne, 2019).

The main activity of VD in the brain was observed in the regulation of calcium signaling, cell proliferation, differentiation, and gene regulation (Alamro et al., 2020; Carlberg and Campbell, 2013; DeLuca et al., 2013; Eyles et al., 2003; Eyles, 2020; Groves et al., 2014; Holick, 2015; Taniura et al., 2006). The researchers found that VD has tendency to cross blood-brain barrier (BBB) in its circulating forms i.e., calcidiol (25-hydroxy VitaminD3 (25-(OH)D3)) and calcitriol (1,25-dihydroxy Vitamin D3 [1,25-

$(OH)_2D3$] and reaches the central nervous system (CNS, Anjum et al., 2023; DeLuca et al., 2013; Galoppin et al., 2022; Mayne and Burne, 2019; Molinari et al., 2019). The active metabolites of VD are calcitriol or $1,25(OH)_2D3$, which binds to the VDR in the brain and modulates the transcription of more than 2000 genes (Carlberg and Campbell, 2013; Warwick et al., 2021). VD belongs to the member of the steroid receptor superfamily. VD-VDR then forms a heterodimer with the retinoid X receptor (RXR), another steroid receptor family member (Chambon, 2005; X. Cui et al., 2017). In the target gene, the VD-VDR/RXR complex binds to the Vitamin D response element (VDRE) and controls the transcription of many target genes in conjoint association with other co-activators and are responsible for the overall genomic effect of calcitriol in the target cell (Bao et al., 2020; Bikle, 2011; X. Cui et al., 2017).

The main resident cells of the central nervous system mainly comprise neurons and glial cells (Barres and Barde, 2000; Brown, 2007; Paridaen and Huttner, 2014; Quan et al., 2022). The beneficial effect of VD in brain occurs mainly to genomic effect which help in neuronal survival, proliferation, and differentiation (Bakhtiari-Dovvombaygi et al., 2021; Di Somma et al., 2017; Evans et al., 2018; Magdy et al., 2022; Manjari et al., 2022). There have also been numerous studies that have shown that VD acts as an as an anti-inflammatory and anti-oxidant agent and aids in glial health (Alessio et al., 2021; Lee et al., 2020; Mazzetti et al., 2022). VD infusion also attenuates microglial activation by decreasing the production of inflammatory cytokines and increasing the expression of Iba-1 (ionized-calcium binding adapter protein-1) which is a sensitive marker for microglia (Calvello et al., 2017; Shi et al., 2021). Similarly, VD regulates the synthesis of different neurotrophic factors like NGF, NT-3, and NT-4 in astrocytes (I. Neveu et al., 1994b, 1994a).

1.4.2. VD: Structure and function

VD is now considered an essential micronutrient, a prohormone, and a neurosteroid with myriad physiological benefits on the skeletal and non-skeletal systems (Anjum et al., 2023; Bendik et al., 2014; Bouillon et al., 2022; Groves et al., 2014; Latham et al., 2021). It can be obtained through various means, like oral supplements, dietary sources, and dermal synthesis (Carpenter and Zhao, 1999; Windans and Bock, 1936). There are two

forms of Vitamin D that are mainly present in nature, i.e., ergocalciferol (Vitamin D2) and cholecalciferol (Vitamin D3), where ergocalciferol (Vitamin D2) is of plant origin, whereas cholecalciferol is of natural animal origin (Zhang and Naughton, 2010). A series of enzymatic reactions takes place within the body converts the inactive precursors of prohormones into active forms. VD enters the body via dietary supplements or sunlight and undergoes a series of enzymatic reactions to form its active form, calcitriol or 1,25-dihydroxycholecalciferol (1α,25-$(OH)_2$D3; Christakos et al., 2016). The active form of VD or calcitriol [1α,25-$(OH)_2$D3] is a secosteroid hormone that contains a broken carbon-carbon double bond at the 9^{th} and 10^{th} position in the B-ring of cyclopentanoperhydrophenanthrene structure which is the basic skeletal structure of steroids as seen in **Fig. 1A** (Seuter et al., 2014). It was reported that the flexible nature of 1α,25-$(OH)_2$D3 structure helps the molecule to bind perfectly into the ligand binding site of Vitamin D binding protein (DBP). Also, the structure of 1α,25-$(OH)_2$D3 helps the molecule to bind to both the nuclear VDR (nVDR) and membrane-bound VDR (mVDR), which further helps in the regulatory function of VD in target organs (Norman et al., 1982). Calcitriol elicits its cellular responses via binding to the VDR. Depending on the mechanism by which it triggers cellular responses, VD shows bowel-like shapes as well as planar-like shapes (Sirajudeen et al., 2019).

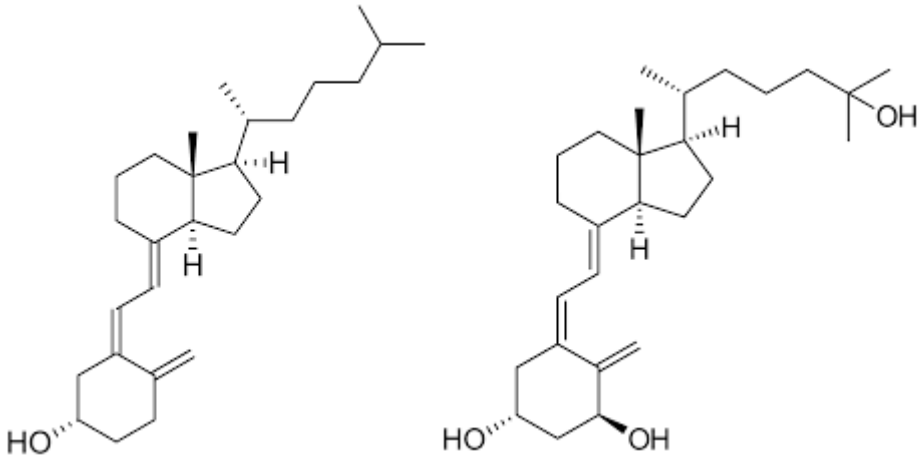

Fig.1. Structure of cholecalciferol and 1α,25$(OH)_2$D3 or calcitriol: The structure of VD is similar to that of steroid, except for the broken rings at positions C9 and C10. Calcitriol is the active metabolite of VD with three (OH) hydroxyl groups and commonly referred to as 1,25-dihydroxycholecalciferol (Image Source: Wikipedia)

There are a number of factors that influence the biological availability of VD, some of which are the season, geographic latitude, time, cloud coverage, and individual characteristics, such as age, pigmentation, and clothing (Webb, 2006). Originally, VD was found to be beneficial for calcium metabolism and the maintenance of bone structure, but it now offers a variety of functional characteristics where it shows its anticarcinogenic, hepatoprotective, cardioprotective, antiarthritic, and neuroprotective properties (Anjum et al., 2018; Eyles et al., 2005; Eyles et al., 2021; Shea et al., 2022). In association with the parathyroid hormone, VD controls calcium and phosphorus homeostasis in the blood, bones, neuromuscular junctions, immune system, and cardiovascular system (Sirajudeen et al., 2019). In the cardiovascular system, it facilitates the renin-angiotensin system, thereby suppressing inflammation in both the heart and the blood vessels (Wang et al., 2008). VD is not only important for the function of peripheral organs but also plays a critical role in the central nervous system (CNS), where higher levels of VD are positively correlated with better memory function and cognition (Anjum et al., 2023; Shea et al., 2023). The latter study reinforces the importance of studying how VD created resilience to protect the aging brain against diseases such as AD and other related dementias (Shea et al., 2023). VD influences a number of important events in brain development, including neurotrophin synthesis, neurotransmitter synthesis, and axonal elongation (Eyles, 2020). VD stimulates NGF, NT-3, and NT-4 production, which are crucial to neuronal survival and development (Cui and Eyles, 2022; Farghali et al., 2020; Gezen-Ak et al., 2014).

Over the last two decades, extensive research on neuronal and non-neuronal brain cells showed that calcitriol not only regulates gene expression but also can rapidly alter the ion channel function (X. Cui et al., 2017). One of the major functional effects of VD was its effect on the function of both voltage-gated and ligand-gated ion channels expressed in the CNS (Restrepo-Angulo et al., 2020). Some of the ion channels recently shown to be modulated directly by calcitriol are L-type voltage-gated calcium channels (L-VGCC), transient receptor potential (TRP) vanilloid family 1 (TRPV1) channel, glutamate-type receptors like N-methyl-D-aspartate (NMDA) and kainate receptors (Long et al., 2021). It is documented that VD induction increased the expression of TRPV6, a TRP channel that augments the calcium uptake in human LNCaP (lymph node cancer of the prostate) cells and thereby enhanced cellular proliferation (Lehen'kyi et al., 2011). Knocking down of

these ion channel receptors inhibited cell proliferation. This suggests that calcitriol regulates the calcium influx by modulating the expression of ion channel receptors (Restrepo-Angulo et al., 2020). Some reports have also showed that modulation in the expression of L-VGCC regulates the secretion of neurotransmitters (Atlas et al., 2001). Interestingly, an opposite effect has been observed in studies on voltage-gated potassium channels like TWIK (Tandem of p domains in a weak inward rectifying potassium channel)-related acid sensitive potassium channel 1 (TASK-1; Callejo et al., 2020).

A significant role is played by VD in brain development through VDR (Erben et al., 2002). Immunohistochemical evidence confirmed the presence of VDR expression in mammalian brains (Dursun and Gezen-Ak, 2017; Liu et al., 2021). VDR is principally located in the nucleus of target cells and requires VDR to show its effect is seen in different regions of the brain like striatum, cortex, hippocampus, amygdala, substantia nigra, and hypothalamus (Cui et al., 2018; Liu et al., 2021; Lv et al., 2020; Moretti et al., 2018; Shah et al., 2019). Most of the vital function of VDR were inferred from studies undertaken in VDR null mice (VDR-/-; Van Cromphaut et al., 2001). These mice showed normal cognition but suffered from abnormal motor behavior, increased anxiety, and also developed hypocalcemia and retarded bone growth (Yoshizawa et al., 1997; Burne et al., 2005; Bouillon et al., 2008). Moreover, gene silencing of VDR using small interfering RNA (siRNA) has been showed to cause a corresponding reduction in neurotrophin production in the normal mammalian brain (Gezen-Ak et al., 2011). In addition, the activation of VDR is known to regulate multiple neuronal signaling pathways through its effect on the expression of BDNF, NGF, and GDNF. The neurotrophins are necessary for the survival, migration and differentiation of developing neurons (Bernd, 2018; Cohen-Cory et al., 2010; Duarte Azevedo et al., 2020; Hall et al., 2018). In one specific finding, authors showed that VDR facilitated c-Ret (c-terminal of rearranged during transfection) mediated downstream signaling pathway and potentiated neuronal survival in the dopaminergic neurons of substantia nigra in a VD deficient rodent model (Pertile et al., 2018). As c-Ret forms the endogenous receptor for the neurotrophin, GDNF, it was inferred from the above studies that VDR can act directly and indirectly via neurotrophins to rescue neuronal survival in multiple brain regions. (Eyles, 2020; Liu et al., 2021).

VDR expression was also observed in the glial cells of the CNS. The two most abundant non-neuronal cells which express VDR are the microglia and the astrocytes (Alessio et al., 2021; Lee et al., 2020; Mazzetti et al., 2022). In microglia, VDR and specific enzymes (1α-hydroxylase) convert calcidiol [25-(OH) D3] to calcitriol ([1,25-$(OH)_2$D3]; Alessio et al., 2021; P. W. Lee et al., 2020). VDR has been reported to be expressed in astrocytes along with the VD activating enzyme i.e., CYP27B1. CYP27B1-positive astrocytes have been showed to display neuroprotective features (Jiao et al., 2017; Mazzetti et al., 2022). Hence, a number of studies are available that confirmed impact of VD on the healthy brain as well as in brain-related disorders (Jang et al., 2015; Mehri et al., 2020; Molnár et al., 2016).

1.4.3. Metabolism of VD:

In late 1930s, the discovery of VD occurred and thereby it was tagged as the "sunshine Vitamin" which had a vital effect on the maintenance of calcium and phosphorus homeostasis (Nair and Maseeh, 2012). The study by Holick and team demonstrated that when 7-dehydrocholesterol in the skin was exposed to ultraviolet light (290-315 nm), pre-Vitamin D3 gets isomerized into Vitamin D3 (Holick et al., 1977; Christakos et al., 2016). The conversion of 7-dehydrocholesterol into pre-Vitamin D3 occurrs within fifteen minutes, which then gets isomerized to produce the active form of VD (calcitriol; Holick, 1981). Calcitriol transportation in the circulation requires a lower binding affinity for Vitamin D binding protein (DBP) to reach its target organs (Hollis, 1984; Horst et al., 1986).

VD metabolism takes palce by following a two steps enzymatic reactions via hydroxylation at the 25^{th} position and hydroxylation at the 1α position in the presence of cytochrome P450 oxidases (CYPs; Sugimoto and Shiro, 2012). These two enzymatic hydroxylation reactions are mandatory to produce the active form of VD. In the first step, cholecalciferol is initially transported to the liver with help of carrier protein where it gets hydroxylated into 25-hydroxyVitamin D or 25(OH)D3 (also called calcidiol) in the presence of the enzyme 25-hydroxylase or CYP2R1 (Cheng et al., 2004). CYP2R1 is the major enzyme responsible for hydroxylation at 25^{th} position of VD (Christakos et al., 2016). The calcidiol or 25-(OH)D3 is then transported to the kidney for its second

hydroxylation step where it is converted into the biologically active form called as 1-alpha-25-dihydroxyVitamin D3 (1α,25-$(OH)_2$D3) or calcitriol by the enzyme, 1α-hydroxylase (cytochrome P450 family 27 subfamily B member 1 or CYP27B1; Christakos et al., 2016). Calcitriol is a potent ligand of the VDR and mediates most of the physiological actions of the VD (Eyles et al., 2005; Eyles et al., 2021). The enzymes, 1α-hydroxylase or CYP27B1 and 25-hydroxylase or CYP2R1 mainly resides in the endoplasmic reticulum or mitochondria and requires cytochrome P450 oxidases for successive hydroxylation reactions and are resident to the inner mitochondrial membrane (Cheng et al., 2003). In the hepatocytes, the majority of the activity of 25-hydroxylase is seen in mitochondria when it binds to CYP2R1 and generates 25-(OH)D3 (calcidiol; Cheng et al., 2003). However, in the kidney, the enzyme, 1α-hydroxylase (CYP27B1) is present both in ER and in mitochondria where the synthesis of calcitriol takes place (Takeyama et al., 1997).

Recent evidence however suggested that the kidney is not the only organ where the second step of hydroxylation takes place to produce calcitriol or (1α,25$(OH)_2$D3) by the enzyme, 1α-hydroxylase (CYP27B1). These enzymes are also present in nonrenal cells like keratinocytes, monocytes, macrophages, osteoblasts, prostate, colon cells, neuron, and glial cells (Bikle, 2011; Jones et al., 2014; Eyles et al., 2005). In the mammalian brain, 1α-hydroxylase (CYP27B1) is found to be widely distributed across different brain regions both during development and in adulthood (Eyles, 2020; Eyles et al., 2005; Gáll and Székely, 2021; Mazzetti et al., 2022; Zehnder et al., 2001). This suggests that neurons and glial cells both can regulate the local production of calcitriol or calcidiol and is capable to modulate neuronal activity (Gáll and Székely, 2021; Eyles et al., 2005; Mazzetti et al., 2022). However, both circulating form of VD (calcidiol and calcitriol) are capable to cross the blood-brain barrier and have the potential to add to the local concentration of calcitriol in the CNS (Anjum et al., 2023).

The catabolism of calcidiol or calcitriol occurs via the mitochondrial enzyme, 24-hydroxylase or 24-hydroxylase (CYP24A1) present in both renal and nonrenal cells (Huang et al., 2015; Christakos et al., 2016). CYP24A1 can catalyze the conversion of both 25-hydroxyVitamin D3 (25-(OH)D3; calcidiol) and 1,25-dihydroxyVitaminD3 (1,25-$(OH)_2$D3; calcitriol) into 24,25-dihydroxyVitamin D3 (24,25-$(OH)_2$D3) which is destined for excretion (Christakos et al., 2016). The primary excretory route for excess VD is

through bile into feces (Jones et al., 2014). CYP24A1 (24-hydroxylase) initiates various catabolic reactions, leading to calcitriol inactivation. The 24-hydroxylation is followed by the oxidation of the 24-OH group to the keto group leading to the cleavage between C23 and C24 positions. Subsequently, this results in the formation of calcitroic acid with no biological activity. Thus, CYP24A1 is likely to play an important role in protecting the body from excess amounts of VD, thereby maintaining sufficient levels of VD in serum (Bikle, 2011; Jones et al., 2014).

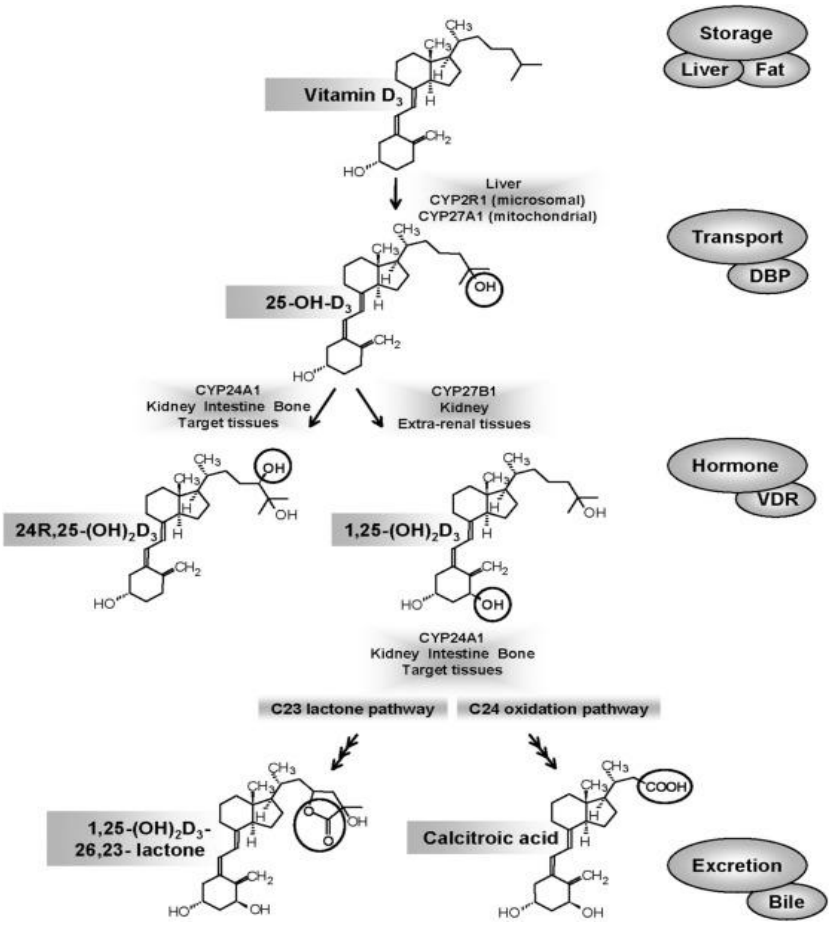

Fig.2. Metabolism of 7-dehydrocholesterol to calcitriol: VD is produced in the skin from 7-dehydrocholesterol in a non-enzymatic process resulting in the formation of pre-Vitamin D which further isomerizes into VD. VD gets converted into 25-hydroxyVitamin D3 in the liver by 25-hydroxylases. The active form of VD i.e., 1α,25-hydroxyVitamin D3 is formed in the kidney by undergoing a second hydroxylation reaction in the presence of 1α-hydroxylases. Whereas, the excess amount of VD gets converted into its inactive form in the presence of 24-hydroxylase to form calcitroic acid. Thus, formed calcitroic acid gets excreted through bile. (Image source: Jones et al., 2014).

1.4.4. Cellular activity of VD:

The cellular effect of the active form of VD or calcitriol is exerted by its binding to its nuclear or membrane-bound VDR (Sirajudeen et al., 2019). The importance of VD in the regulation of different aspects of brain development, differentiation and cellular machinery was defined via the degree of expression of VDR (Anjum et al., 2023; Di Somma et al., 2017; Moretti et al., 2018; Umar et al., 2018). Due to the differential gene expression of VDR in different brain regions like striatum, cortex, hippocampus has differential gene expression of VDR, there is a region specific effect of VD-VDR mediated benefits on neuronal function (Cui and Eyles, 2022; Landel et al., 2017; Liu et al., 2021).

1.4.4.1. Structure and function of VDR – Cellular localization:

VDR is found mainly in mammalian tissues where its binding to the active metabolite of VD i.e., calcitriol regulates the expression of more than 2000 genes (Carlberg and Campbell, 2013; Warwick et al., 2021). It was proposed that the activity of VDR takes place through either cell signaling pathways or by ligand-activated transcription factors, depending on whether the receptor is membrane-bound or nuclear bound (Bikle, 2021; Rastinejad et al., 2013; Sirajudeen et al., 2019; Zhang et al., 2011; Zmijewski and Carlberg, 2020).

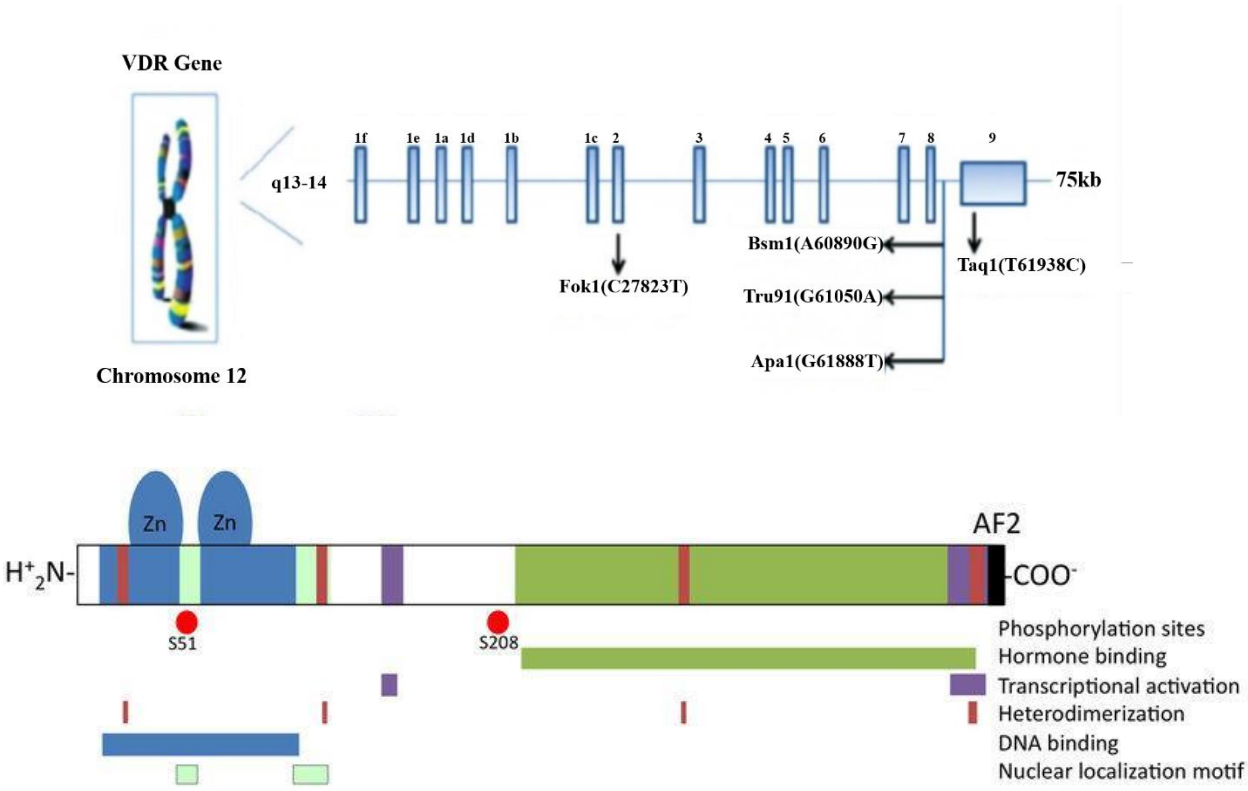

Fig.3. Schematic representation of chromosomal and protein domains of nVDR: On the 12q chromosome, VDR is located. VDR protein contains several functional domains. Two zinc finger motifs are found at the N-terminus of the DNA binding domain and nuclear localization motif. A hormone-binding domain is found at the protein's C-terminus, which also contains an activation function (AF-2) domain, while heterodimerization regions are found in the scattered regions of the protein, where it binds to the retinoid X receptor. (Image source: Fibla and Caruz, 2010, Manchanda et al., 2012)

The gene encoding for VDR is located on the 12th chromosome (Bid et al., 2012). The molecular mass of VDR is 48 kilodalton with two functional domains namely, a DNA-binding domain (DBD) and a ligand-binding domain (LBD).

(i) **DNA-binding domain (DBD):** In the DNA-binding domain of VDR, there are two zinc finger motifs which is located near the N-terminus region of the protein (Fibla and Caruz, 2010). The first zinc finger is important for the formation of heterodimer with retenoid X receptor (RXR) whereas the second zinc finger is involved in binding of specific DNA to the Vitamin D response element (VDRE) of the target gene (Rochel, 2022; Wan et al., 2015). RXR binds to the upper half site while VDR binds to the downstream site of VDRE which are separated by three base pairs of

spacer DNA (DR3; Shaffer and Gewirth, 2002). DNA binding of VDR is dependent upon the availability of the binding site through pioneer factors and coactivators, which opens the chromatin and modify chromatin topology (Rochel, 2022).

(ii) **Ligand-binding domain (LBD)**: The ligand-binding domain is located at the C-terminal region of VDR, along with a motif necessary to activate the function of VDR which is called ligand-dependent transcriptional activation domain (AF-2; Fibla and Caruz, 2010). LBD domain contain twelve helices, i.e., three two turn helices and three-stranded beta sheets which undergoes conformational changes when bound to its ligand (Rochel, 2022). The binding of specific agonist (calcitriol) with VDR induces conformational change in the first 12 helices which are buried deep inside the receptor allowing the upregulation of transcriptional activity of different target genes (Mutchie et al., 2019; Rochel, 2022; Wan et al., 2015; Zmijewski and Carlberg, 2020). The gene expression of target genes ultimately depends on the conformational changes induced in the 12^{th} helix which recruits coactivator at the beginning of the gene expression (Rochel, 2022). The absence of agonist causes no conformational change in the 12^{th} helix, thereby inhibiting the recruitment of coactivator which in turn suppresses gene expression. It is suggested that AF-2 motif may mediate transactivation through ligand-dependent intermolecular interaction with coactivators and through ligand-induced intramolecular contacts with the VDR ligand-binding domain itself. This balance in the conformational changes in 12^{th} helix of LBD that is the major regulator for transcription regulation of many genes in the target cells (Ekimoto et al., 2021; Rochel, 2022; Slominski et al., 2021; Wan et al., 2015).

Calcitriol mediates its biological effects by binding with VDRs via genomic and non-genomic pathways, both of which are manifest through nuclear VDRs (nVDRs) and membrane-bound VDRs (mVDRs; Fibla and Caruz, 2010, Manchanda et al., 2012).

1.4.4.2. Genomic action of VDR:

The genomic activity of VD is facilitated through its binding with the VDR located in the nVDR (Lu et al., 2018; Sirajudeen et al., 2019; Zhang et al., 2023). It is attributed by the interaction between VD, VDR, and retinoid X receptors (RXR). When calcitriol gets attached to VDR, it undergoes conformational changes in the ligand binding domain. These conformational changes facilitate the formation of heterodimer with RXR. The VD-VDR/RXR complex interacts with the VDRE in the promoter region of target genes across the genome to induce or suppress the gene expression. The classical form of VDRE consists of two half-sites separated by three nucleotides, referred to as DR3 (Shaffer and Gewirth, 2002). The VD-VDR/RXR complex can also bind to the DR4 type of VDRE (Carlberg and Dunlop, 2006). When the VD-VDR/RXR complex binds with VDRE, it leads to the release of co-repressor proteins, such as nuclear receptor corepressor 2 or silencing mediator of retinoic acid and thyroid hormone receptor (NcoR2/SMRT), in addition to recruiting co-activators, such as steroid receptor coactivator 1 (SRC1), that promote the expression of target genes (Haussler et al., 2013). When the VD-VDR/RXR complex binds to the promoter region of the specific gene, it initiates transcription, where the rate-limiting step is based on the the bio availability of calcitriol or VD (Eyles, 2020). The genomic effect of VD occurs in nearly all the body cells. For the purpose of this thesis, we will mostly focus on the genomic effect of VD in developmental, adult, and aged brain.

Majority of the studies which reflect the genomic activity of VD on neurogenesis focused on the hippocampal region. Several groups have investigated the effect of VD on the developing hippocampal neurons, neuronal outgrowth, and neuronal development via regulating the gene expression of NGF (Brown et al., 2003; X. Cui et al., 2017; Juwita et al., 2021; Marini et al., 2010; Morello et al., 2018; I. Neveu et al., 1994a; Ucuz et al., 2015). Gene array analysis showed that low levels of VD during gestation influence the genes involved in neuronal development and differentiation via the upregulation of myriad neurotrophic factors (Brown et al., 2003; Naveilhan et al., 1996; I. Neveu et al., 1994a). Many insights on the genomic effect of VD also were inferred from studies utilizing a developmental VD (DVD) deficient or 1α-hydroxylase lacking enzyme rodent models (Groves and Burne, 2017). VD deficiency in pregnant females decreases the neurosphere

formation and reduces cognitive function in the offspring, suggesting that VD plays a vital role in cell proliferation (Chowdhury et al., 2020; Cui et al., 2007). There was also an increase in cell proliferation in the hippocampal dentate gyrus and a decrease in the survival of newborn neurons (Morello et al., 2018). According to a study on SH-SY5Y cells, calcitriol is associated with GDNF expression via VDR, which is critical for the development of dopaminergic neurons (Pertile et al., 2018). Using the neonatal brain of the DVD rat, it has been determined that VD is essential to neurotransmitter release, which will be necessary to transfer chemical signals from one neuron to another and to facilitate synaptic plasticity (Kesby et al., 2017; Lovinger, 2010). The findings of all these studies suggest that VD may be directly associated with neural expansion, differentiation, and development.

VD also promotes neurogenesis in the adult hippocampus. Several in-vitro and in-vivo studies have shown that VD supplementation increases the density of hippocampal neurons in adults (Brewer et al., 2001; Buell and Dawson-Hughes, 2008; Landfield and Cadwallader–Neal, 1998; Latimer et al., 2014; Moretti et al., 2018). Several studies on VD that suggested that the level of VD is directly related to the activity and expression of neurotrophins like BDNF, NGF, NT-3, NT-4, and GDNF in the brain (Eyles, 2020; Groves and Burne, 2017; Lardner, 2015; Nadimi et al., 2020; Pertile et al., 2018). NGF is thought to be one of these neurotrophins that are mainly expressed by neurons in the hippocampus and neocortex, where it is primarily involved in neuronal survival, neurotransmission, and synaptic function (Farghali et al., 2020; Liu et al., 2018). According to a study, the administration of calcitriol directly to the hippocampus of adult rats showed an increased expression of NGF, which is helpful for the survival of the adult brain (Saporito et al., 1993). Another neurotrophic factor is GDNF, which is necessary for the differentiation and survival of dopaminergic neurons (Allen et al., 2013; Lara-Rodarte et al., 2021; Pertile et al., 2018). According to a study on neural stem cells isolated from the hippocampus of 8-week-old mice, calcitriol supplementation increased the proliferation rate by increasing the expression of BDNF, NT-3, and GDNF (Shirazi et al., 2015). The effects of calcitriol supplementation on primary neural progenitor cells were observed to increase Ki^{+} and proliferating cell nuclear antigen (PCNA) expression, thereby directly stimulating proliferation (Morello et al., 2018). Some studies showed that calcitriol supplementation

inhibits calcium influx in mesencephalic and hippocampal neurons by lowering the expression of L-VGCC and increasing the expression of Calbindin D-28K and Na^{+}/Ca^{2+} exchangers (Alexianu et al., 1998; Brewer et al., 2001; Gezen-Ak et al., 2011; Ibi et al., 2001). Along with neurogenesis and development, calcitriol also acts as a protective agent by decreasing reactive oxygen species (ROS) through the Nrf2 (nuclear factor erythroid 2 related factors) regulatory network, controlling antioxidant expression and suppressing neuroinflammation by regulating the expression of inflammatory cytokines (Calton et al., 2015; Calvello et al., 2017; Garcion et al., 1998; Lefebvre d'Hellencourt et al., 2003).

According to the studies, it is believed that there is a close relationship between the levels of calcidiol and changes in the level of neurotransmitters, which help to carry chemical insights from one neuron to another (Farhangi et al., 2017; Patrick and Ames, 2015; Seyedi et al., 2019). As a result of calcitriol supplementation to cortical neurons isolated from embryonic rats at 18 days old, synaptic proteins like synapsin-1 were increased, and glutamate toxicity decreased with time, indicating that calcitriol maintains synaptic plasticity (Taniura et al., 2006). In different brain regions, such as the hippocampus and prefrontal cortex, VD is associated with significant changes in levels of dopamine, serotonin, acetylcholine, gamma-aminobutyric acid (GABA), and glutamate due to its genomic regulation of their respective rate-limiting enzymes (Groves et al., 2014; Jiang et al., 2014; Pertile et al., 2016).

Evidence suggests that the genome-wide effect of calcitriol can be attributed to the presence of the target gene's promoter region on the VDRE, which regulates neurotrophins, oxidative stress, inflammation, synaptic transmission, and the activity of ion channels in the brain (Brown et al., 2003; Cohen-Cory et al., 2010; Haussler et al., 1998; Naveilhan et al., 1996; I. Neveu et al., 1994a). In this way, VD supplementation may directly modulate the differentiation, maturation, and survival of neurons. In addition to its effect on neurotrophins, VD supplementation also maintains oxidative stress. It has been found that VD supplementation in a developing brain stimulates the expression of nitric oxide (NOS) and the activity of gamma-glutamyl transpeptidase and glutathione (GSH) in the rat hippocampal tissues (Garcion et al., 1998). Furthermore, VD protects cells from oxidative stress by increasing the expression of antioxidants like superoxide dismutase (SOD) and catalase in rats exposed to mild stress (Bakhtiari-Dovvombaygi et al., 2021). To determine

the anti-oxidant effect of VD, researchers investigated traumatic brain-injured rats that showed a decrease in NADPH oxidase expression with an increase in VDR expression, which determined the antioxidant effects of calcitriol by its genomic action (Jamilian et al., 2019).

However, there have been numerous studies linking low levels of VD with age-related neurodegenerative disorders such as AD, PD, and HD (Chai et al., 2019; Chel et al., 2013; Fullard and Duda, 2020). The evidence suggested that treating different neurological disorders, such as AD and PD, with VD caused a profound increase in BDNF production, which corresponded to the findings regarding the development of BDNF (AlJohri et al., 2019; Bayo-Olugbami et al., 2022; Lima et al., 2018; Mohamed et al., 2015). In another study, VD was shown to reverse age-induced memory deficits and maintain synaptic plasticity by altering the expression of BDNF, NGF, TrKA, and TrkB in the hippocampal tissues of rats (Bayat et al., 2021). It has been demonstrated in some preclinical studies that calcitriol enhanced cognitive functioning and memory in age-related neurological conditions such as AD and PD (Anjum et al., 2023; Koduah et al., 2017; Latimer et al., 2014; Morello et al., 2018). The calcitriol also plays a protective role in AD and PD models by regulating neurotrophins, anti-oxidants, and inflammation cytokines, similar to embryonic stages (Calvello et al., 2017; Landel et al., 2016; Lima et al., 2018; Saad El-Din et al., 2020a; Y. Zhang et al., 2022). According to clinical studies, VD can improve posture, balance, and memory in elderly AD and PD patients with an increase in serum concentrations of 25-(OH)D3 (Chakkera et al., n.d.; Fullard and Duda, 2020; Hiller et al., 2018; Jia et al., 2019). According to this analysis, there is a positive correlation between the levels of VD and different neurodegenerative disorders. Researchers have determined that VD may promote healthy aging of the brain through its influence on synaptic function (Almeras et al., 2007; Eyles et al., 2007; Latimer et al., 2014; Mayne and Burne, 2019; Wang et al., 2023). According to a study on middle-aged rats, VD regulates synaptic proteins involved in synaptic vesicle trafficking and neurotransmission (Latimer et al., 2014). Additionally, calcitriol administration induced a reduction in the density and activity of the L-VGCC in hippocampal neurons of older rats, which is thought to retard calcium influx, which may contribute to preventing some of the toxic outcomes associated with neurodegenerative diseases like AD and PD (Behl et al.,

2022; Brewer et al., 2006; Gezen-Ak et al., 2011; Ibi et al., 2001). The data gathered by the researchers suggest that VD promotes the development and survival of neurons in the adult brain by delaying the process of aging.

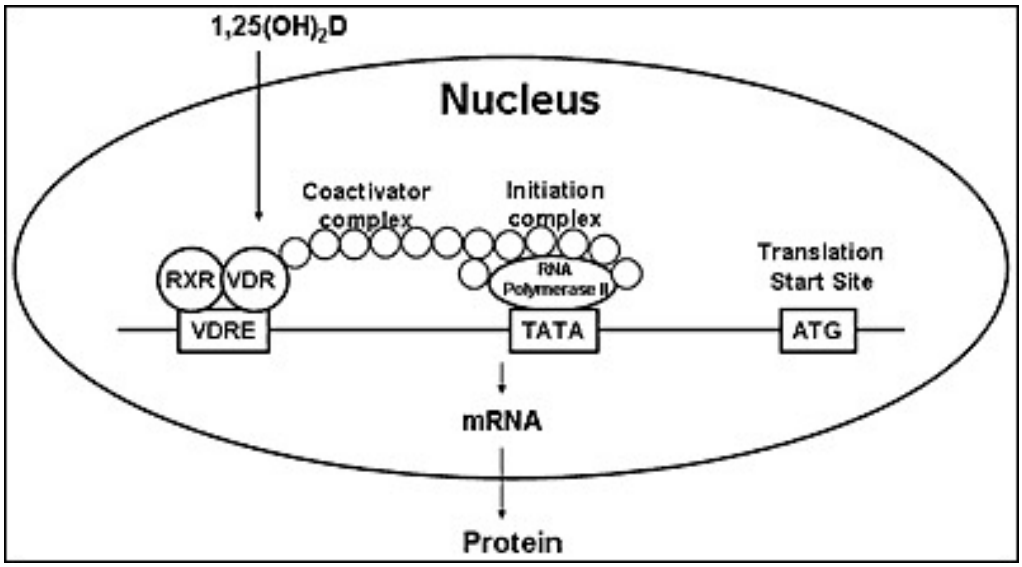

Fig.4. Genomic pathway of VD: As soon as 1α,25-$(OH)_2$D3 binds to its VDR receptor, it initiates gene transcription, which then heterodimer with RXR, which increases the affinity of the VD-VDR/RXR for specific promoter regions. Co-activators tend to bind to VDRE when the VD-VDR/RXR complex binds to the VDRE, which recruits RNA polymerase II to the TATA box. By transcribing genes, mRNA is produced and translated into specific proteins by RNA. (Image source: Bikle, 2021)

1.4.4.2.1. Epigenetic modifications due to the activation of VDR:

The interaction between calcitriol and VDR not only results in genomic effects but also leads to epigenetic changes (Fetahu et al., 2014; Karlic and Varga, 2011). The mechanism of transcriptional regulation of VDRE and VDR activation involves histone modification, chromatin remodeling, and an alteration in the binding of RNA polymerase II (Di Rosa et al., 2011; Ramagopalan et al., 2010). It was found that the VD-VDR complex plays an important role in transiently opening and closing chromatin at RNA polymerase II-enhanced sites, which determines the regulation of the primary target genes for the calcitriol (Carlberg and Molnár, 2015). According to these studies, calcitriol helps to regulate genes via epigenetic mechanisms and, conversely, regulates epigenetic events (Bahrami et al., 2018; Fetahu et al., 2014; Xue et al., 2016). The primary epigenetic

modifications caused by calcitriol include acetylation and histone protein methylation (Nurminen et al., 2018; Sawatsubashi et al., 2019). Calcitriol induces DNA methylation at the promoter site of the target genes, which can be repetitive over a period of several DNA replication cycles (Doig et al., 2013). Due to this modification by calcitriol, there is an alteration in the accessibility of DNA and chromatin structure that can lead to aberrant gene expression, which is ultimately linked to the development and progression of many fatal diseases (Anderson et al., 2018; Beckett et al., 2016; Fetahu et al., 2014; Lai et al., 2020; Xue et al., 2016). Although this may be the case, calcitriol still regulates DNA methylation to ensure its proper functioning. In addition to the methylation process, supplementation of calcitriol also aids in acetylation. The interaction between the VD-VDR/RXR complex and the histone acetyltransferases (HATs), which introduce acetyl groups into the nucleosomes, resulting in the chromatin being more accessible to transcription factors (Karlic and Varga, 2011). Evidence shows that VDR can bind to DNA without a ligand and form complexes with histone deacetylases (HDACs; Liu et al., 2017; Seuter et al., 2014). In addition, it appears that VD supplements cause the acetylation of histone 3 acetylation at lysine 27 (H3K27), which is associated with activated enhancers in the genome of target genes (Sawatsubashi et al., 2019; Seuter et al., 2014). This acetylation or methylation of H3K27 could possibly be regarded as part of a mechanism that allows the opening or closing of chromatin (Sawatsubashi et al., 2019). In addition, it has been shown that VDR-mediated regulation of targeted gene expression can be carried out through the modification of HATs and HDACs (Pike and Meyer, 2012; Wang, 2007). The acetylation and deacetylation of histones lead to the opening and closing of chromatin, regulating the transcription of target genes (Eberharter and Becker, 2002; Gujral et al., 2020). Calcitriol also helps to deacetylate H3 and histone 3 dimethylated lysine 9 (H3K9) di-methylation (Fu et al., 2013). The process of acetylation and methylation contributes to regulating gene expression (H.-T. Lee et al., 2020; Miller and Grant, 2013). These epigenetic modifications in the presence of the VD-VDR/RXR complex involve the differentiation and formation of cells by transcription regulation.

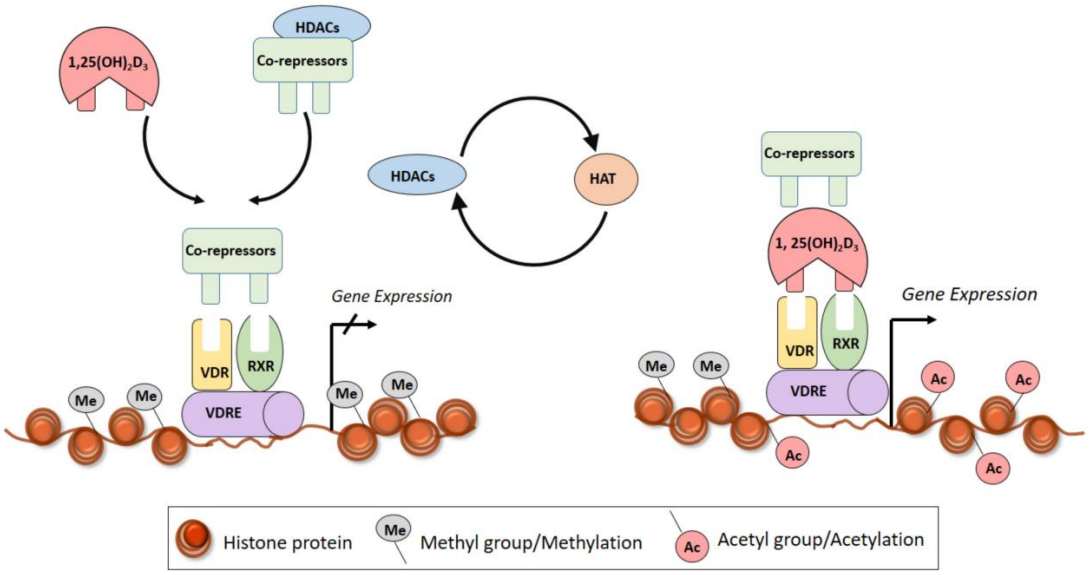

Fig.5. Schematic illustration of histone modification by methylation and acetylation mediated by calcitriol and its link with the basic transcription machinery: Cells package DNA in the heterochromatin region, resulting in a dense array of nucleosomes. The tightly packed chromatin is inaccessible to transcription factors. Through protein-protein interactions with co-activators with HAT activity, the chromatin opens. In order to promote gene expression and relax chromatin structure, two major epigenetic modifications, DNA methylation and histone modification, are essential. VDR is inactivated and expressed less when DNA methylation occurs at its promoter region. Histone deacetylases (HDAC) corepressors bound to heterochromatin regions suppress downstream gene expression by repressing transcriptional machinery, which leads to downstream gene silencing. VDR-RXR complexes bind with HATs, which cause histone acetylation. In this way, transcription factors can be recruited, and downstream genes can be expressed. (Image source: Krishna, 2019)

1.4.4.3. Non-genomic action of VD:

The non-genomic actions of 1α,25-dihydroxyVitamin D3 or 1α,25-$(OH)_2$D3 or calcitriol occur in many peripheral cells, including the central nervous system (Bollen and Atherton, 2021; X. Cui et al., 2017; de Angelis et al., 2017; Hii and Ferrante, 2016; Shirvani et al., 2019; Zmijewski and Carlberg, 2020). Compared with genomic actions, non-genomic actions of VD involve mechanisms independent of nVDR activation and are more rapid. VD propagates the non-genomic action by binding to the membrane-bound VDR (mVDR) on the cell surface (Sirajudeen et al., 2019). Furthermore, co-immunoprecipitation studies have shown that 1,25-membrane-associated rapid response

steroid-binding proteins (1,25-MARRS) also interact in non-nuclear compartments. As a result, both mVDR and 1,25-MARRS are essential for the non-genomic actions of VD. They function as chaperone proteins and are involved in a number of downstream signal transduction pathways (Chen et al., 2013, 2010). Protein disulfide isomerase 3 (PDIA3), which is also known as endoplasmic reticulum stress protein 57 (ERp57), is an additional receptor that is expressed by all types of brain cells involved in the non-genomic action of VD, along with mVDR and 1,25-MARRS (Landel et al., 2017; Sequeira et al., 2012). A study showed that 1,25D3-MARRS/Pdia3/ERp57 receptor activation reduced the number of amyloid plaques and removed neurofibrillary tangles in the cortex and hippocampus of 5XFAD mice with AD (Tohda et al., 2012). There have been numerous studies that suggest that calcitriol binding to mVDR caused the activation of calcium and kinase signaling pathways, which activate a variety of protein kinases, including phosphoinositol-3 kinase (PI3K), protein kinase A (PKA) and calmodulin-dependent protein kinase II (CamKII; Christakos et al., 2016; Gooch et al., 2019; Zanatta et al., 2012). The action of these kinases further activates secondary signaling molecules such as phospholipase C (PLC), phospholipase A2 (PLA2), mitogen-activated protein kinase (MAPK), extracellular signal-related protein kinase 1 and 2 (ERK1/2) and G protein-coupled receptors (Bikle, 2021; de Angelis et al., 2017; Habib et al., 2020; Hii and Ferrante, 2016; Vuolo et al., 2012; Zaulkffali et al., 2019). The downstream signaling pathways of these kinases are directly or indirectly related to transcriptional regulation via the cross-talk between the genomic effects of VD through nVDRs that can aid in the survival of cells (de Angelis et al., 2017). Interestingly, it is notable that most of the intracellular molecules that participate in the non-genomic pathways are common to different target organs (Bollen and Atherton, 2021; de Angelis et al., 2017; Donati et al., 2022; Hii and Ferrante, 2016). There is also an evidence showed that the non-genomic effects of VD were due to its binding to an alternative ligand binding pocket in classical nVDRs (Mizwicki et al., 2010). Generally, it is accepted that the non-genomic action of VD signaling facilitates the genomic action by stimulating the phosphorylation of RXR or other transcription factors (J. Chen et al., 2013; Zanello and Norman, 2004). The findings made with VDR knockout monocytes indicate that the non-genomic actions of VD modulate transcriptional responses were not independent of VDR (Warwick et al., 2021). Despite this, different signaling cascades are

involved in determining the non-genomic effect of VD in a range of cell types, as well as at various stages during the development of each cell type and point in time (Doroudi et al., 2012; Zamoner et al., 2008; Zanatta et al., 2012, 2011).

According to Cui and colleagues, calcitriol is involved in calcium and kinase signaling pathways that regulate cell survival. Furthermore, VD modulates synaptic transmission by increasing the frequency of it in juvenile gonadotropin-releasing hormone (GnRH) neurons. This study showed that acute application of calcitriol decreased the inward currents induced by the excitatory NMDA and kainite receptors (Bhattarai et al., 2017). Furthermore, calcitriol rapidly reduces the frequency of GABAergic postsynaptic currents. As a result of all these changes, VD may maintain the balance between excitation and inhibition of local neural circuits in the brain (Bhattarai et al., 2017).

The non-genomic ativtiy of VD also gets propagated via the regulation of ion channels (Cui and Eyles, 2022). The L-type voltage-gated calcium channels (L-VGCC) facilitate the electrical signaling of cells by allowing restricted passage of calcium ions inside the cells in response to changes in the membrane potential of the cell. It is shown that a suboptimal concentration of VD could alter brain maturation through modulation of L-VGCC signaling (Zanatta et al., 2012).

The rapid non-genomic action of VD increases calcium influx in the cortex and prefrontal cortex, which ultimately maintains the Ca^{2+} homeostasis in these areas by the cross-talk between the genomic and non-genomic actions of VD on L-VGCC and protects these cells from excitotoxicity (Cui at al., 2017; Cui and Eyles, 2022; Zanatta et al., 2012). In response to the activation of L-VGCC, a number of protein kinases, such as protein kinase C (PKC), protein kinase A (PKA) and mitogen-activated protein kinase-extracellular signal-regulated kinase-cascade-1 and 2 (MAPK-ERK-1/2) are phosphorylated, acting as secondary messengers (Gooch et al., 2019; Gravielle, 2021; Subbamanda and Bhargava, 2022). The phosphorylation of these protein kinases play a vital role in the cross-talk with genomic effects, which leads to transcription regulation, cell growth, and differentiation, as well as apoptosis, as depicted in **Fig. 6** (Vuolo et al., 2012).

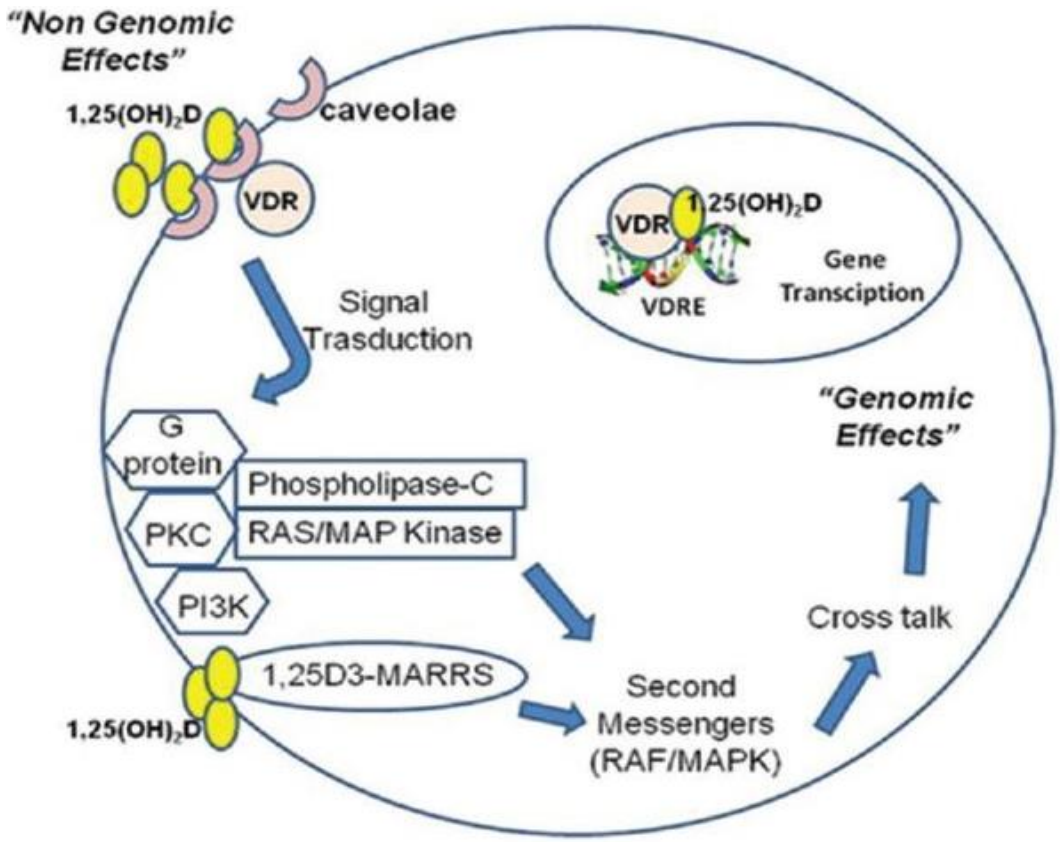

Fig. 6: Non-genomic action of VD: VD exerts non-genomic effects by binding membrane-bound VDR (mVDR). Several intermediate factors are activated when vd binds with mVDR, which eventually activates MAPK-ERK-1/2, activating VD's non-genomic action. Calcium influx is stimulated by activated VDR, resulting in a cascade of intracellular pathways like protein kinase C (PKC). At an intracellular level, VD activates G protein-coupled receptors (GPCRs), which lead to several signaling pathways, including phospholipase C (PLC) and phosphatidylinositol 3-kinase. As a result of the convergence of these pathways, MAPK-ERK-1/2 is activated, which further interacts with VDR genomic action and modulates gene expression. (Image Source: Vuolo et al., 2012)

Besides activating L-VGCC, VD also activates K^+ and Cl^- channels, which regulate membrane potential through Na^+/K^+-ATPase (X. Cui et al., 2017). Further, the non-genomic actions of VD include the opening up of channels for Ca^{2+} and Cl^- as well (Bikle, 2021; Gooch et al., 2019; Norman, 2006). Since most of the nongenomic activity is focused on signaling pathways and ion channel function, it may have modulatory effects on the innate and adaptive immune responses of cells as well as cell survival (X. Cui et al., 2017; Cutolo et al., 2023; Hii and Ferrante, 2016). These responses may include interaction between mVDR and target proteins like IkappaB kinase (IKKB), which regulates the

activity of NF-κB, signal transducers and activators of transcription (Stat1), runt-related transcription factor (RunX), c-jun, β -catenin and cREB which further regulate the survival pathways (Y. Chen et al., 2013; Hii and Ferrante, 2016; Lange et al., 2014; Nadimi et al., 2020; Pálmer et al., 2001; Wei and Christakos, 2015). Some mitochondrial functions are directly or indirectly affected by VD, including energy production, mitochondrial membrane potential, ion channel activity, and apoptosis (Zmijewski and Carlberg, 2020).

Therefore, VDR activation may promote neuronal survival through interactions between genomic and non-genomic pathways. As a result of this cross-talk, neurotrophic factors, like BDNF, are upregulated along with autophagy, and antioxidant pathways are activated (Abdulghani et al., 2023; Zhao et al., 2022). An intricate network of these pathways is responsible for repairing and promoting neuronal damage in a wide range of neuropathological conditions, as well as promoting their survival.

1.4.4.4. Activity of VD on different neurotrophins:

One of the primary effects of VD in the maintenance of brain development and function is through its regulation of the expression and activity of key neurotrophins in the CNS (Anjum et al., 2023; Cui and Eyles, 2022; Lv et al., 2020). VD activates a variety of neurotrophins, including NGF, BDNF, NT-3, NT-4, and GDNF (Gezen-Ak et al., 2014; Khairy and Attia, 2021; Koshkina et al., 2019; Lv et al., 2020; Nadimi et al., 2020; Pertile et al., 2018; Pignolo et al., 2022). Neurotrophins (BDNF, NGF, GDNF, NT-3, NT-4) play an essential role in neuron growth, development, neurotransmission, and synaptic plasticity (Bathina and Das, 2015; Li et al., 2016; Pertile et al., 2018; Proenca et al., 2016; Wang et al., 2020). Because of their versatile activity in normal brain function, alteration in the expression of different neurotrophins has been studied in neurological disease conditions and correlated with degeneration of neurons. Under different neuropathological conditions like AD, PD, depression, and HD, VD has been found to regulate the activity and expression of neurotrophins (Alamro et al., 2020; Chunmei Geng et al., 2019; Koshkina et al., 2019; Mohamed et al., 2015; Pignolo et al., 2022).

Across all the brain regions, VD has been shown to enhance the expression of BDNF, NGF, NT-3, and NT-4, while an increase in the expression of GDNF is seen in

specific brain regions like substantia nigra (Cornet et al., 1998; Naveilhan et al., 1996; Neveu et al., 1994; Saporito et al., 1994).

The primary evidence gathered by Neveu and team showed that, in glial cells and astrocytes, calcitriol enhances the expression of neurotrophic factors such as NGF, NT-3, and NT-4 (Isabelle Neveu et al., 1994; Wion et al., 1991). An initial study determined that calcitriol administration to hippocampal neurons of adults induced NGF expression (Saporito et al., 1993). In a study by Gezen-Ak and colleagues, silencing the VDR genes using siRNA in primary cortical neurons decreased NGF release, which determined that VD increases the levels of NGF through VDR and helps in neuronal survival (Gezen-Ak et al., 2011). Later, it was determined that an increase in VDR expression increases the release of NGF, which further prevents beta-amyloid elevation and toxicity (Gezen-Ak et al., 2014). Different studies have also found increased levels and expression of NGF on VD supplementation (Alamro et al., 2020a; Koshkina et al., 2019; Pignolo et al., 2022). The activity of NGF is mediated by TrkA receptors, which help to stimulate cell growth and prevent programmed cell death (Minnone et al., 2017). TrkA receptors undergo autophosphorylation of their tyrosine residues when NGF binds with them (Marlin and Li, 2015). Phosphorylation of these receptors activates PI3K, Akt kinases. The downstream targets of these kinases are essential for neuronal survival (Yan et al., 2020). Furthermore, autophosphorylation of TrKA receptors leads to the activation of MAPK through a Ras-mediated pathway (Reichardt, 2006). As a result of MAPK activation, ERK1/2 is phosphorylated, which further regulates cyclic adenosine monophosphate response element binding protein (CREB), ultimately leading to neuronal survival (Koga et al., 2019). Beside from the two pathways described above, phosphorylation of TrkA receptors activates phospholipase C (PLC) and protein kinase C (PKC) to promote neuronal survival. Thus, VD-mediated transcription plays a role in regulating the expression of NGF, which is critical for neuronal survival (Reichardt, 2006).

According to a study by Naveilhan and colleagues, VD can induce BDNF production and regulate expression of BDNF in C6 glioma cells (Naveilhan et al., 1996). Further, Atif and coworkers demonstrated that calcitriol supplementation increases the protein expression of BDNF in ischemic stroke-induced cortical cells (Atif et al., 2013). Their study found that supplementing cells with calcitriol increased the gene expression of

BDNF thereby increasing the protein expression of TrkB receptors (Massa et al., 2010). As a result, downstream signaling of ERK1/2 is activated, which then transduces growth factor signals to the nucleus, affecting gene expression. ERK1/2 phosphorylation contributes to metabolism, mitosis, differentiation, inflammation, cell death, and survival (Lu and Xu, 2006). Study findings suggested that BDNF has a neuroprotective effect when expression of ERK1/2 is increased in the cells since ERK1/2 induces expression of anti-apoptotic genes such as BCl-2 (Atif et al., 2013; Nilsen and Brinton, 2002). Calcitriol supplementation to neural stem cells (NSCs) increased the expression of neurotrophic factors like NT-3, BDNF, and GDNF, which promote oligodendrocyte proliferation and differentiation (Shirazi et al., 2015). According to a study on adult rats subjected to stress, calcitriol increased the levels of neurotrophins like BDNF, NT-3, and NT-4 in their brains (Koshkina et al., 2019). The results of this study supported the prescription of VD supplements for treating depression in females. Similarly, VD supplementation to diabetic rats increased the levels of BDNF in hippocampal tissues. According to their study, BDNF increased TrkB receptor phosphorylation, activating transcription factor CREB, which are important for neuronal survival (Gabryelska et al., 2023; Nadimi et al., 2020). Based on the findings from recent clinical studies, it has been showed that when VD is supplemented to patients with depression, the levels of BDNF in the serum were significantly increased (Abiri and Vafa, 2020; Paduchová et al., 2021). Additionally, recent studies have found that supplementing with VD increased the levels of BDNF in hippocampal tissues of animal models of different neurological conditions (Bakhtiari-Dovvombaygi et al., 2021; Xu and Liang, 2021).

According to different studies, VD regulates the expression of other neurotrophins like NT-3 and NT-4 (Koshkina et al., 2019; Shirazi et al., 2015). The activity of NT-4 gets mediated by TrkB receptors, which is similar to the downstream signaling pathway involved in the action of BDNF (Proenca et al., 2016). NT-3, on the other hand, initiates its activity by binding to TrkC receptors. When NT-3 binds to TrkC receptors, the receptors undergo phosphorylation thereby triggering the intracellular signaling pathway (Houlton et al., 2019; Khan and Smith, 2015). It is anticipated that the downstream signaling pathway of the phosphorylated TrKC receptors will be carried out through the ERK1/2

pathway, which helps in neuronal differentiation, as well as the PI3K pathway, which further leads to neuronal survival (Khan and Smith, 2015; Yan et al., 2021).

GDNF is another neurotrophin that gets modulated on a cellular level following VD supplementation, which helps mainly in the survival of dopaminergic neurons (Cortés et al., 2017; Lara-Rodarte et al., 2021; Lin et al., 1993; Sariola and Saarma, 2003). Dopaminergic neurons benefit from GDNF downstream signaling through Ret receptors, which are highly expressed in the substantia nigra (Gattei et al., 1997; Kramer et al., 2007; Mahato and Sidorova, 2020; Stanga et al., 2018). Homodimers of GDNF aggregate two Ret molecules containing tyrosine residues, resulting in the accumulation of GDNF (Bahlakeh et al., 2021). In response to the transphosphorylation of that tyrosine residue, intracellular cascades will get triggered, including MAPK and PI3K pathways, which are critical to neurite survival and neurite outgrowth (Allen et al., 2013). The above evidence altogether supports the idea that VD plays a significant role in the regulation of neurotrophins, which play a crucial role in the survival, proliferation, and differentiation of neurons in the brain.

1.4.4.5. Antioxidant role of VD–Organelle-based function:

The redox system plays a critical role in maintaining cellular homeostasis by generating or eliminating ROS (Le Gal et al., 2021; Schieber and Chandel, 2014; Trachootham et al., 2008). The main types of ROS are oxygen free radicals (O_2^-), hydroxyl radicals ($OH^{\cdot}$), and hydrogen peroxide (H_2O_2; Collin, 2019; Munnamalai and Suter, 2009). Usually, decrease in the amount of molecular oxygen leads to the production of ROS. The redox system is usually responsible for controlling gene transcription through cysteine residue binding to DNA transcription sites and epigenetic modifications through the remodeling of chromatin (Rhee, 2006). Moreover, it contributes to post-translational modifications that lead to conformational changes in peptides due to oxidative alterations of amino acid residues, which help regulate protein structure and function (Petushkova and Zamyatnin, 2020). Moreover, recent evidence suggested that the redox system played a novel role in regulating cell proliferation and survival by post-translational modification of signaling proteins, ubiquitin proteosomes, and other proteases. Cross-talk between redox

regulatory pathways governing survival and death is responsible for this (Foyer et al., 2018; Kriegenburg et al., 2011; Schieber and Chandel, 2014; Trachootham et al., 2008).

An imbalance in the redox system in the cells is caused by the overproduction of ROS, which overwhelms the protective and defense mechanisms that help protect the cells from various physiological conditions (Berg et al., 2004). This imbalance will result in hypoxia, mitochondrial dysfunction, protein misfolding, inflammatory responses, lipid peroxidation, DNA damage, and interference of ROS with signal transduction pathways. Additionally, this process will also involve the degradation of proteins, which play a major role in maintaining cellular health (Franco-Iborra et al., 2016; Rey et al., 2023; Rotermund et al., 2018). When redox system is imbalanced, the production of oxidized proteins and lipids increases. This is considered an early event in the progression of neurodegenerative diseases (Andersen, 2004). It has been found that the production of ROS plays a role in the progression of a variety of neurodegenerative disorders such as AD, PD, and HD (Agnihotri and Aruoma, 2020; O'Regan et al., 2021; Zhang et al., 2020). VD has received considerable attention as a redox imbalance management tool due to its antioxidant properties. Whether VD directly affects the disposal of free radicals or indirectly by activating essential neuroprotection pathways is a much-debated question. This area is not fully studied in the pathologies of different neuronal diseases. As an evidence of calcitriol blocking ROS production and preventing their hydroxyl donors, there was an evidence of its direct impact (Ibi et al., 2001).

Some antioxidants, such as superoxide dismutase (SOD), glutathione peroxidase (GpX), Catalase (Cat), nitric oxide synthase (NOS), and nitrogen oxide (NOX), are regulated by VD, which regulates mitochondrial function by ROS (Calvello et al., 2017; Cui et al., 2019; da Costa et al., 2023; Patel and Shah, 2022; Y. Zhang et al., 2022). In preliminary studies of calcitriol supplementation in different neurological conditions, it has been showed that this treatment had a beneficial effect on reducing NOS and gamma-glutamyl transpeptidase, thereby increasing the amount of glutathione (GSH; E. Garcion et al., 2002; Garcion et al., 1998). Based on a study on cultured rat cortical neurons, it has been determined that supplementation with calcitriol reduces glutamate toxicity by increasing the expression of VDR (Taniura et al., 2006). VD supplementation has been found to increase the gene expression of antioxidants like GSH, SOD, and GpX by binding

to VDRE (Brown and Slatopolsky, 2008). It has been recognized that GSH is one of the major endogenous components of the cellular defense system, which is specifically responsible for scavenging ROS directly (Galano and Raúl Alvarez-Idaboy, 2011). There is evidence that antioxidants that help to prevent oxidative stress react with glutathione and act against free radicals. Therefore, it can be speculated that increase in glutathione levels will help regulate cell proliferation (McCarty and DiNicolantonio, 2015). A study on traumatic brain-injured rats showed that VD treatment decreased NOX activity in hippocampal tissues (C. Cui et al., 2017). It has been found that VD reduces lipid peroxidation, which influences the activity of antioxidants (AlJohri et al., 2019). VD is shown to have antioxidant activity via the VDR that inhibits the expression of multiple antioxidant enzymes, such as NOX2, NOX4, and the NADPH oxidase (Cui et al., 2019). According to a clinical study on patients suffering from psychiatric disorders, supplementation with VD lead to improvement in total antioxidant capacity (TAC) and glutathione (GSH) and remarkable decrease in the levels of MDA (Jamilian et al., 2019). VD can have an antioxidant effect in the brain as a result of an increase in oxidative stress, which results in an upregulation of the expression of CYP27B1, a brain-resident enzyme, leading to the formation of calcitriol in the brain (Huang et al., 2015; Hur et al., 2014; Kasatkina et al., 2020). A study conducted with a rat model of AD demonstrated that long-term administration of VD can slow down excessive oxidative stress by enhancing the activity of SOD (Mehrabadi and Sadr, 2020). The enzyme SOD plays a vital role in the functions of the antioxidant defense system by catalyzing the dismutating of oxygen-free radicals into molecular oxygen and hydrogen peroxide (Younus, 2018). There are a variety of possible mechanisms through which VD reduces oxidative stress. One of them is that it boosts levels of enzymes that protect brain tissue from oxidative stress, such as SOD and thiol, which reduces MDA, one of the markers of oxidative stress, in brain tissue samples (Bakhtiari-Dovvombaygi et al., 2021b; Bayo-Olugbami et al., 2022; Khairy and Attia, 2021). In 2018, Lima and colleagues reported that VD supplementation induced dopamine metabolism in mice induced with 6-hydroxydopamine (6-OHDA), which helps to preserve motor function and regulate lipid peroxidation (Lima et al., 2018). Further, studies have demonstrated that supplementing with VD lowers the appearance of oxidative stress markers by increasing the expression of Sirt1 (Sirtuin 1), which is important in preventing

mitochondrial damage in various neurological diseases like PD, AD, ALS, and HD (Chen et al., 2005; Hasegawa and Yoshikawa, 2008; Kim et al., 2007; Magdy et al., 2022; Pallàs et al., 2008; Wareski et al., 2009). The pretreatment with calcitriol significantly reduced the levels of lipid peroxidation and the release of apoptotic proteins in iron-treated locus coeruleus of rats (Mello-Filho and Meneghini, 1991). Moreover, the calcitriol administration also prevented the oxidation and accumulation of ferric iron in the neuronal cells (Chen et al., 2003). Similarly, calcitriol was also found to reduce ROS formation and cell death in cortical neuronal cells exposed to Zinc (Lin and Beal, 2006). It has been found that VD treatment could enhance the activity of Nrf2, a transcription factor well known for the synthesis of antioxidant enzymes (C. Cui et al., 2021). As a result, all the above evidence demonstrates the efficacy of VD as an antioxidant in treating neurological diseases.

1.4.4.6. Anti-neuroinflammatory role of VD:

Inflammation in neuronal cells is a major factor contributing to the onset and progression of neurodegenerative diseases (Hou et al., 2019). The process gets triggered by the overactivation of innate immune response cells, such as microglia or astrocytes, in response to protein misfolding or other environmental stress that diverts from the beneficiary function to sustained release of pro-inflammatory molecules (Bachiller et al., 2018; Blach-Olszewska and Leszek, 2007; Novellino et al., 2020; Rotermund et al., 2018). The antioxidant effects of VD are well documented, but many studies have demonstrated that the substance can slow the inflammatory cytokine storm that antagonizes neuronal loss (Banerjee et al., 2015; Calvello et al., 2017; Cannell, 2008; Groves et al., 2014; Wang et al., 2023).

Initially, Lemire and Archer found that calcitriol has an anti-inflammatory effect in the relapsing model of EAE, the MS model. When the lymphocyte proliferation and IL-2 expression were decreased in the brain, they concluded that VD might had immunomodulatory role (Lemire and Archer, 1991). Garcion and colleagues later demonstrated in lipopolysaccharide-induced cells that calcitriol inhibits induced nitric oxide synthase (iNOS) expression in rat brain monocytes. According to this study, VD supplementation reduces the effects of immune-induced oxidative stress in the CNS

(Garcion et al., 1998). Later, a study on EOC microglial cell line showed that calcitriol administration had inhibited the activity of tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) by increasing the expression of VDR and increased the level of transforming growth factor-beta subtype 1 (TGF-β1; Lefebvre d'Hellencourt et al., 2003). According to another study, treatment with calcitriol significantly decreased the expression of TNF-α and interferon-gamma (IFN-γ) in microglia from rats treated with toxic-induced PD models of the disease. The immunomodulatory effects of VD have been established by decreasing the gene expression of these two proteins in microglia (Kim et al., 2006).

Recent studies have showed that VD exerts its protective effects by modulating the activity of TNF-α, IL-1β, IL-6, TGF-β, and IL-10 in conditions such as aging and cognitive decline (Calvello et al., 2017; Cui et al., 2019; Evans et al., 2018; Patel and Shah, 2022). The fact that multiple sclerosis is considered a chronic inflammatory disorder has led to numerous studies demonstrating that people with adequate VD are at a relatively low risk of becoming ill with this disease (Dörr and Paul, 2015; Munger et al., 2004). On this note, a study on the toxin induced mouse model of PD has shown that VD supplementation decreased the expression of pro-inflammatory cytokines like TNF-α, and IL-1β and increased the expression of anti-inflammatory markers like IL-10, TGF-β, and IL-4 in both striatum and substantia nigra (Calvello et al., 2017). Furthermore, the study found that VD administration reduced the expression of CD206 (cluster of differentiation 206), CD163 (cluster of differentiation 163), and CD204 (cluster of differentiation 204) in the microglia of PD mice (Calvello et al., 2017). The results of an investigation conducted by Wang and his colleagues in 2018 determined that serum levels of VD were negatively correlated with the IL-6, a proinflammatory marker (Wang et al., 2018). In the same year, Evans and team found that acute VD administration reduced the expression of pro-inflammatory markers like interleukin-21 (IL-21) and interleukin-23a (IL-23a) in ischemic stroke patients, indicating that VD regulates inflammatory responses in the peripheral organs and the brain, thereby maintaining brain health (Evans et al., 2018). The evidence showed that VD supplementation improves spatial and working memory in rats induced with lipopolysaccharide by decreasing the levels of TNF-α, IL-1β, and TGF-β1 (Doncheva et al., 2022). It has also been showed that the active form of calcitriol modulated the phenotype of T cells by inhibiting JAK-STAT signaling, which further increased the

expression of T helper cells (Magdy et al., 2022). Apart from its ability to affect different pro- and anti-inflammatory cytokines, VD has also been showed to facilitate the differentiation of M2 cells by stimulating the synthesis of toll-like receptors 4 and 10 (TLR-4 and TLR-10), which in turn leads to an intracellular signaling pathway that activates innate immunity (Calvello et al., 2017; Verma and Kim, 2016). Calcitriol also has a neuroinflammatory role in lipopolysaccharide-induced pericytes by modulating the expression of genes such as CCL2 (chemokine (c-c motif) ligand 2), CHRDL1 (chordin like 1), KLF4 (Kruppel like factor 4), TNFPAIP6 (tumor necrosis factor-alpha-induced protein 6), TNFS4 (tumor necrosis factor superfamily number 4), and VCAM (vascular cell adhesion molecule 1). According to the results of this study, calcitriol can be used to prevent or treat neuropsychiatric disorders (Nissou et al., 2014). Furthermore, VD is known to have a direct effect on the expression of proinflammatory cytokines in damaged neurons, microglia, and astrocytes (Cui and Eyles, 2022; Galoppin et al., 2022; P. W. Lee et al., 2020). Another study on PD showed that VD supplementation attenuated neuroinflammation in microglia by downregulating the expression of TLR-4 and upregulating the expression of anti-inflammatory cytokines like IL-10, IL-4, and TGF-β (Calvello et al., 2017). Studies have shown that astrocytes can also express CYP27B1 and VDR (Jiao et al., 2017; Lu et al., 2018; Mazzetti et al., 2022). Therefore, supplementing astrocytes with VD will boost the production of calcitriol and suppress inflammation. A recent study involving lipopolysaccharide-induced astrocytes found that VD administration suppressesd the expression of pro-inflammatory cytokines like TNF-α, IL-1β, and TLR-4, thus reducing astrocyte activation (Jiao et al., 2017). Conversely, the microglia are considered a key player in different inflammatory and neurodegenerative disorders (Bachiller et al., 2018; Rodríguez-Gómez et al., 2020). There is a possibility that calcitriol can be synthesized by microglia because of the presence of the enzyme CYP27B1 (Boontanrart et al., 2016; Smolders et al., 2021). Various studies on neuroinflammatory models like multiple sclerosis, it was demonstrated that VD could reduce the activation of microglia and antigen-presenting cells by downregulating the expression of Iba1, MHC-II (Major histocompatibility complex II), CD86 (cluster of differentiation 86), and TLR-4 (Galoppin et al., 2022). Furthermore, VD treatment has decreased the production of pro-inflammatory cytokines like IL-6, TNF-α, and IL-1β and, on the other hand, increased the

expression of anti-inflammatory cytokines like TGF-β1, IL-10, IL-4, IFN-α and IFN-β in microglia in different models in different neuroinflammatory models including EAE (Galoppin et al., 2022). Collectively, it may be proposed that VD could be used as a valuable tool due to affirmative action on neuro-inflammation observed in neurodegenerative diseases.

1.4.4.7. Anti-cholinesterase activity of VD in the brain:

The cholinergic system plays an essential role in arousal, learning, memory, and attention (Huang et al., 2022; Teles-Grilo Ruivo et al., 2017; Villano et al., 2017). A neurotransmitter released by cholinergic neurons, acetylcholine (ACh), which acts as a chemical messenger and allows them to communicate with one another (Picciotto et al., 2012). The synthesis of Ach is carried out at the axon terminals from two components, choline, and acetyl-CoA, in the presence of choline acetyltransferase (Aboughazala and Anan, 2020; Bellier and Kimura, 2007). Once the action potential reaches the axon terminus, acetylcholine is released into synaptic clefts through the vesicles (Han et al., 2017; Sugita et al., 2016). The breakdown of ACh occurs in the synaptic cleft because of the presence of acetylcholinesterase (AChE; McHardy et al., 2017). Ach has been shown to promote cytoskeleton organization, cell proliferation, differentiation, and apoptosis through the activation of its two types of receptors i.e., muscarinic acetylcholine receptor (mAChRs) and nicotinic acetylcholine receptors (nAChRs; Dani, 2015; Liu et al., 2015; Mashimo et al., 2019; Resende and Adhikari, 2009; Zoli et al., 2018). The receptors are multisubunit proteins derived from both neuromuscular and neuronal tissues that form ion channels that are ligand-gated and play a role in the transmission of synaptic signals between neurons and neuromuscular junctions (Dani, 2015; Papke and Lindstorm, 2020; Unwin, 2013; Zoli et al., 2018). In the CNS, neuronal nAChRs are mostly expressed in presynaptic, postsynaptic, and extra-synaptic regions (Dani, 2015; McKay et al., 2007; Resende and Adhikari, 2009). Among these α7 nAChRs are mostly expressed in the regions implicated with cognition, learning, and memory, such as the neocortex and hippocampus, and the levels are low in the striatum, forebrain, and various brain nuclei (Cao et al., 2022). When ACh binds to the α7 nAChRs, it causes confirmation changes in the receptors, leading to the efficient exchange of ions, particularly with Ca^{2+} and thereby

promoting downstream signaling cascades, which are responsible for controlling a variety of neurotransmitter releases, cell survival, and cellular excitability processes (Cao et al., 2022; Cheng and Yakel, 2015; Mashimo et al., 2019; McKay et al., 2007; Zoli et al., 2018). These α7 nAChRs are homopentameric in nature, which gets distinguished from other nAChRs due to their low affinity binding with α-Bungarotoxin (α-BTX), which are highly expressed in presynaptic and postsynaptic sites of CNS. Homopentameric α7 nAChRs contain an extracellular domain at their N-terminus, a small extracellular domain at their C-terminus, and four transmembrane domains connected by cytoplasmic loops. The receptor is dysfunctional in neurological disorders such as Alzheimer's disease, Parkinson's disease, schizophrenia, and multiple sclerosis. Such neurological conditions will impair nAChR function due to impaired energy and glucose metabolism in the brain (Jackson-Guilford et al., 2000). Thus, it may provide a novel therapeutic target for diseases like Alzheimer's disease, Parkinson's disease, schizophrenia, and multiple sclerosis that alter cholinergic signaling.

Initial studies determine that VD regulated the expression of neurotransmitters like ACh as well as the rate-limiting enzymes involved to maintain the levels of ACh like choline acetyltransferase and acetylcholinesterase (Emmanuel Garcion et al., 2002; Sonnenberg et al., 1986). However, a study had shown that VD supplementation normalized the nAChR dysfunction, which lowered the time of spatial recognition and improved cognitive functions (Kumar et al., 2011). It was found that VD supplementation decreased the cholinergic activity in the prefrontal cortex of scopolamine-induced AD rats and streptozotocin-induced diabetic rats by reducing the activity of AChE, thus rescuing the learning and memory (Alrefaie and Alhayani, 2015; Karabulut et al., 2021). Similarly, another study on the hippocampus of aged mice and diabetic rats showed that VD levels were positively correlated with the activity of choline acetyltransferase and decreased the level of AChE (Al-Zahrani et al., 2021; Khairy and Attia, 2021; J. Zhang et al., 2022). Another study using a nicotine withdrawal mouse model showed that VD supplementation improved the expression levels of all α7 nAChRs in the hippocampus, influencing memory, cognitive function, and synaptic transmission (Wu et al., 2021). In a recent study, it has been demonstrated that VD improves the cholinergic activity in the cortex of AD rats by decreasing the activity of AChE and increasing the concentrations of ACh (Rodrigues et

al., 2019). Overall, the results demonstrate that cholinergic interventions are more prevalent in the cortex compared to the striatum since α7 nAChRs in the latter are less common.

1.4.4.8. Anti-apoptotic effect of VD in the central nervous system:

The mechanism of apoptosis is the energy-dependent cascade of molecular pathways that occurs in almost all neurodegenerative diseases (Chi et al., 2018; J. Cui et al., 2021; Moujalled et al., 2021). The two types of apoptotic pathway mechanisms were studied in stress conditions. First is the mitochondria-mediated pathway, known as the intrinsic pathway, and the other is a receptor-mediated pathway, known as the extrinsic pathway, which involves an energy-dependent cascade of molecular pathways (Elmore, 2007; Radi et al., 2014). In most neurological diseases, neurons undergo apoptosis via intrinsic pathways, which can be induced by neurotrophin deprivation, DNA damage, mitochondrial dysfunction, or endoplasmic reticulum stress (Hollville et al., 2019; Maity et al., 2022; Shadfar et al., 2022; Shen et al., 2020). The stimulation of these stressors activates specific signaling events by reducing Akt signaling, resulting in the activation of anti-apoptotic and pro-apoptotic proteins that maintain mitochondrial membrane integrity (Ambacher et al., 2012; Green and Llambi, 2015; Hollville et al., 2019). Through a cascade of intracellular pathways, these pro-apoptotic proteins, such as Bcl-2 associated X protein (Bax), form an oligomer and insert into outer mitochondrial membranes, releasing cytochrome c into the cytosol (Garrido et al., 2006; Hussar, 2022; Peña-Blanco and García-Sáez, 2018). In response to Cyt-c binding to Apaf-1, an apoptosome is formed, which activates caspase-9 (Bratton and Salvesen, 2010; Jiang and Wang, 2000). An activated caspase-9 triggers a series of proteins that lead to DNA fragmentation and chromatin condensation, leading to apoptosis (Elmore, 2007; Faleiro and Lazebnik, 2000).

Consequently, VD has the ability to reduce apoptosis and neuronal death by downregulating the expression of L-VGCC and upregulating the expression of membrane Ca^{2+}-ATPase, thereby predicting its role in neuronal survival (Eyles et al., 2013). Low levels of the VDR protein have been linked to impair autophagy, resulting in a decrease in the number of functional genes involved in regulating apoptosis (Uberti et al., 2014). A study on the EAE mouse model of multiple sclerosis revealed that VD supplementation

increased B-cell leukemia/lymphoma 2 protein (Bcl-2)/Bax, thereby inhibiting apoptosis (Zhen et al., 2015). A study on traumatic brain injury showed that VDR activation suppressed neuronal apoptosis, which determined that VDR acts as a self-defensive protein to overcome pathological stress conditions (C. Cui et al., 2017). These studies shoed that VD can rescue apoptotic cell death by activating the VDR. VD supplementation gained attention for its widespread anti-apoptotic role in different brain regions, leading to reduced reactive oxygen species (Yamini et al., 2018). This indicates the relationship between VD deficiency and impaired cognitive function. Previous studies on AD showed that VD supplementation decreased the number of apoptotic bodies and increased the number of viable cells in hippocampal and cortical tissues, respectively (Alamro et al., 2020a; Yamini et al., 2018). A study conducted on different aged matched in vitro cultures demonstrated that VD supplementation decreased the expression of cytochrome c, a crucial regulator of energy metabolism and apoptotic pathways associated with mitochondrial dysfunction (Molinari et al., 2019).

In the same way, an evidence determined that VD administration to seizure-induced rats decreased expressions of c-fos, Bax, and caspase-3, thereby preventing the apoptosis of hippocampal cells (Şahin et al., 2019). As per reports from recent studies, VD is found to regulate nuclear factor kappa B (NF-κB), Bcl-2, Bax, and caspase-3, which are listed as risk factors in age-related neurological disorders such as AD and PD (Bao et al., 2020; Bayo-Olugbami et al., 2022; Khairy and Attia, 2021; Lin et al., 2020; Magdy et al., 2022). The VD supplementation resulted in a reduction in the expression of Bax, C-Fos, and Caspase-3 levels in AD and hyperthyroidism, which may have a positive effect on apoptosis in the brain, especially in the hippocampal region (Rastegar-Moghaddam et al., 2023). Overall, these results indicate that VD has a vital function in regulating apoptosis during neurological conditions, demonstrating its anti-apoptotic properties.

1.4.4.9. VD helps to maintain synaptic plasticity:

Synaptic plasticity involves the generation of new synapses, eliminating synapses if not required, and altering the structural, molecular, and electrophysiological properties of existing synapses based on their functionality (Chelini et al., 2018; Citri and Malenka, 2008). A synapse consists of pre-synaptic terminal loaded with neurotransmitter vesicles

and postsynaptic compartment with an array of receptors to receive a response from neurotransmitters, which leads to synaptic plasticity (Citri and Malenka, 2008). Synaptic plasticity plays a vital role in the development and maintenance of neural circuitry, and accumulating evidence suggests that impairment in synaptic plasticity contributes to different neurological disorders (Perez-Catalan et al., 2021). Depending on the stimulus or various other conditions like drug administration, synaptic transmission can either be enhanced or depressed, and these changes may last from milliseconds to hours or days or even longer (Almeras et al., 2007; Eyles, 2020; Latimer et al., 2014). Overall, a synapse consists of pre-synaptic terminal loaded with neurotransmitter vesicles and postsynaptic compartment, which is packed with receptors to receive response from neurotransmitters. An alteration in the molecular mechanism of synaptic transmission or changes in the surrounding environment is considered to be synaptic dysfunction (Ardiles et al., 2017). This synaptic dysfunction and degeneration is regarded as a common hallmark for neurological diseases like AD, PD, and HD (Lepeta et al., 2016; Marsh and Alifragis, 2018; Taoufik et al., 2018). These diseases are characterized by an imbalance in the composition, organization, and function of synaptic terminals caused by protein aggregates. Protein aggregates are involved in different mechanisms through which they bind to synaptic proteins, such as cellular prion protein, post-synaptic density protein 95 (PSD95), synaptosomal associated protein 25 (SNAP 25), neurogranin, synaptotagmin-1 and synapsin 1 (Agnello et al., 2021; Baker et al., 2018; Laurén et al., 2009; Levy et al., 2022; Mirza and Zahid, 2017; Smith et al., 2007). In addition, various studies showed that prenatal VD deficiency may alter genes involved in synaptic plasticity, primarily debrin, and neuromodulin.

In light of this, it is evident that VD plays an important role in the maintenance of synaptic transmission, as well as modulating synaptic plasticity (Gáll and Székely, 2021). There is mounting evidence that VD plays an essential role in long-term potentiation (LTP) in the brain, which is a widely recognized mechanism of synaptic plasticity and an essential component of information storage in the brain (Eyles, 2020; Latimer et al., 2014). In addition, VD administration in aged rats increased synaptic plasticity by increasing the expression of genes involved in synaptic plasticity, such as synaptojanin 1, synaptotagmin 2, and calcium/calmodulin-dependent protein kinase (Latimer et al., 2014). According to

another evidence on AD, VD supported nerve transmission and synaptic plasticity by detecting extracellular postsynaptic potentials (Taghizadeh et al., 2014). The researchers reported that VD administration increased the protein concentration of synapsin and PSD-95 when the neurons were exposed to glutamate-induced neurotoxicity, which is important for the development and maturation of dendritic spines and synaptogenesis (Kouba et al., 2023; Taniura et al., 2006).

In another study, L-VGCC upregulation maintained nitric oxide (NO) levels, which play a key role in neurotransmission, synaptic plasticity, and neuroprotection. Nevertheless, it is already known that the optimal level of VD maintains L-VGCC expression and activity (Hölscher, 1997; Murdaca et al., 2021). According to electrophysiological measurements on hippocampal brain regions, supplementing with VD improved synaptic plasticity by cellular excitability in aged rats (Bayat et al., 2021). According to research, VD has a role in the release of neurotransmitters like dopamine, serotonin, and glutamate and in the expression of their corresponding receptors, both of which are essential to synaptic function (Eyles, 2020; Mayne and Burne, 2019; Wang et al., 2023). Recent evidence has demonstrated that supplementation with VD regulated some synaptic proteins such as synaptophysin, SNAP-25, PSD-95, and synapsin-1 (Liang et al., 2018; Mutchie et al., 2019; Wang et al., 2023). These studies suggest that VD supplementation regulates neuronal function and relieves age-related cognitive decline.

1.4.5. VD deficiency:

VD deficiency has increased exponentially in recent years. However, the definition of VD deficiency is still under debate. Most of the clinical data suggest that the serum concentration of 25(OH)D3 depicts the bioavailability of VD in the body to carry out different biological functions (Holick and Chen, 2008). Moreover, when the concentration of 25(OH)D3 in the serum is >30ng/ml, it is considered sufficient, whereas if the attention is 21-29 ng/ml, it is considered insufficient. When the serum concentration is less than 20 ng/ml, that condition is considered VD deficiency (Dawson-Hughes et al., 2010).

1.4.5.1. Global and Indian prevalence of VD deficiency:

Deficiency of VD is one of the world's most serious public health problems. The problem affects all age groups, even in tropical countries. Several clinical evidences

indicate more cases were reported in North America and Europe than in other regions (Gois et al., 2017). In elderly populations, VD deficiency is more prevalent due to reduced sunlight exposure, reduced dietary intake, and reduced ability of the skin to produce 7-dehydrocholesterol, a component that contributes to VD formation (Gois et al., 2017). Compared with European countries, clinical data from Middle-Eastern countries also showed low levels of VD in serum, i.e., 10-14ng/ml (Kaykhaei et al., 2011; Sayed-Hassan et al., 2014).

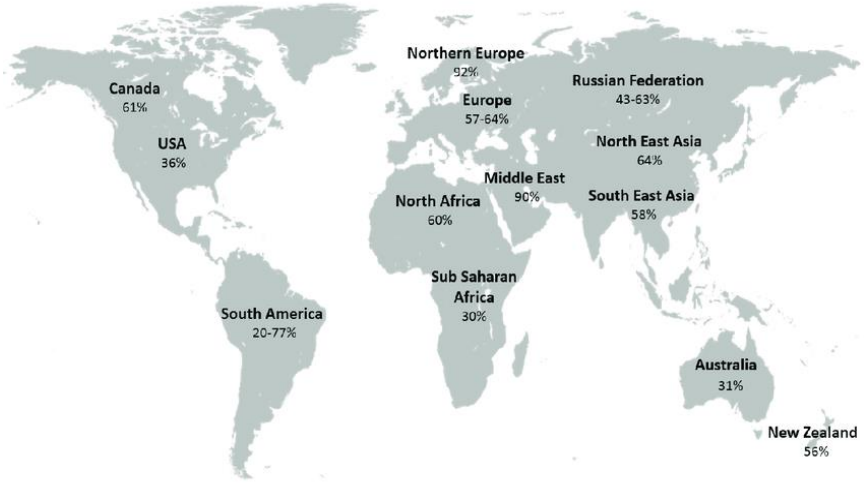

Fig. 7. Global prevalence of VD deficiency: The prevalence of VD deficiency studies worldwide has increased in different regions as a growing public health problem that might change over time. (Image source: Gois et al., 2017)

It has been estimated from clinical evidence that 68% of people from South Asia were deficient in VD, representing 24% of the global population (Siddiqee et al., 2021). Due to the fact that India is one of those tropical countries where people are exposed to sunlight all the time and thus should be able to get enough VD, it has been showing a gradual increase in VD deficiency compared to other countries in South Asia (Aparna et al., 2018). According to reports, three out of four people have this condition. As a diversified country, India also has varying levels of VD deficiency based on age, location, food, and cultural habits. The reason for this is likely the vegetarian eating habits of people, and animal foods are considered a rich source of VD. According to clinical studies, VD deficiency will be an epidemic in India by 2022, affecting over 70% of the population (Khadilkar et al., 2022).

1.4.5.2. VD deficiency in different neurological conditions:

Hypovitaminosis D3 is seen in patients with peripheral diseases such as type 1 diabetes mellitus, inflammatory bowel disease, anemia, rickets, osteoporosis, rheumatoid arthritis, etc. Additionally, previous studies have found that low levels of VD are linked to neurological conditions like AD, PD, MS, Amyloid lateral Sclerosis, schizophrenia, and HD (Chel et al., 2013; X. Cui et al., 2021; Di Somma et al., 2017). A similar pathway is associated with these neurological diseases, including inflammatory responses, oxidative stress, mitochondrial dysfunction, DNA damage, and synaptic dysfunction. As a result of all these conditions, neuronal death occurs, resulting in cognitive decline and memory loss (Wilson et al., 2023). A ligand, calcitriol, enhances the expression of VDR, which regulates neurotrophins, oxidative stress, neuroinflammation, and synaptic strength, thereby promoting cell survival and neurotransmission.

Furthermore, VD deficiency disrupts the balance between excitatory and inhibitory neurotransmitters. Based on research on mice and rat models, it was shown that VD deficiency decreases glutamate production and increases levels of gamma-aminobutyric acid (GABA), which results in altered behavior and cognition (Groves et al., 2013; Kasatkina et al., 2020). As a consequence of this imbalance between excitatory and inhibitory neurotransmitters, there could be an increase in reactive oxygen species production and calcium levels at the nerve terminals (X. Cui et al., 2021).

Several in vitro studies had also found that VD can regulate cytotoxicity, protein misfolding, apoptosis, and inflammation (Banerjee and Chatterjee, 2003). Additionally, studies had found that VD deficiency may cause deficits in brain development. According to meta-analyses, patients with VD deficiency exhibit cognitive impairment, a common phenotypic feature in different neurological disorders (Goodwill and Szoeke, 2017). Despite these findings, it is evident that VD regulates gene expression that contributes to cytoskeleton maintenance, calcium homeostasis, synaptic plasticity, neurotransmission, oxidative phosphorylation, mitochondrial function, protein transport, chaperone maintenance, cell cycle regulation, as well as post-translational modifications that are altered by VD deficiency (Almeras et al., 2007; Eyles et al., 2007; Eyles, 2020). Based on this evidence, we can conclude that VD deficiency contributes to the decline of

neurocognitive function and the accelerated process of neurodegeneration. This determines that VD deficiency contributes significantly to early brain aging (Terock et al., 2022). The MRI scan of elderly VD deficient patients showed reduced hippocampal volume and deficits associated with the right hippocampal lobe. This disrupts the network, resulting in cognitive impairment (Al-Amin et al., 2019). Low serum levels of calcidiol are associated with decreased mitochondrial respiration, which contributes to a lack of energy. It increases oxidative stress, which may contribute to neurodegenerative diseases related to aging (Berridge, 2017; Kim et al., 2014). There is evidence that VD deficiency accelerates the process of aging, leading to aging-related neurological disorders.

1.4.6. The activity of VD in different neurological conditions:

In the nervous system, VD protects neurons against multiple risk factors caused by various neurological conditions. When patients live with neurological disorders, VD supplementation can be crucial in restoring VD homeostasis and preventing neurological diseases (X. Cui et al., 2017; Landel et al., 2016). To minimize the risk of such conditions, the endocrine society suggested specific doses of VD to reach a minimum level of calcidiol, i.e., 30ng/ml in the serum. Depending on the baseline level of calcidiol, the environmental conditions, and the age of the patient, the dosage of VD will be determined (Holick et al., 2011). Thus, based on the results of the previous study, it was evident that depending on the baseline level of calcidiol, an optimized dose of VD supplementation can have a therapeutic impact on different neurological diseases (Moretti et al., 2018). Therefore, VD supplementation might be safe and inexpensive for treating age-related neurological disorders. The cellular effects of VD might be able to rescue the risk factors and improve the pathologies of diverse neurological conditions.

1.4.6.1. The dose-dependent effect of VD in different neurological conditions:

Based on recommendations and case studies from different endocrinologist societies, VD supplementation dosage varies according to baseline serum calcidiol levels, responsiveness, and disease severity (Di Somma et al., 2017). The risk of VD deficiency and the interaction with the specific type of neurological illness require routine monitoring of VD levels in the blood. In response to serum calcidiol levels, the dosage of VD will be determined through diet or external supplementation (Anwar et al., 2023). According to

clinical studies, almost 70-90% of people with neurological conditions like AD, PD, MS, schizophrenia, and HD have a VD deficiency. VD can have a wide range of effects, which vary with different doses and depending on the conditions in which it is used as follows:

1.4.6.1.1. The effect of different doses of VD in different clinical conditions:

Initially, neurological conditions like MS was seen with a VD deficiency, for which endocrinologists have tried different doses of VD with limited success. An initial study conducted on MS showed that dietary supplements containing ≥400 IU/day of VD daily decreased the risk of developing the disease by 40%, increasing the serum level of VD by 20ng/ml (Munger et al., 2006, 2004). According to the clinical studies by Annweiler and Beauchet's, patients with moderate AD conditions showed improvements when supplemented with a high dose of VD, i.e., 10,000IU each month, after which they have improved their language, memory, and cognitive abilities, which further reveals that prevention of neuronal damage (Annweiler and Beauchet, 2011; Annweiler, 2016). On the other hand, in a randomized survey of placebo-controlled participants, it was found that supplementing with 400 IU of VD did not significantly enhance cognitive function (Rossom et al., 2012). In a study of patients with AD who were supplemented with a low dose of VD, i.e., ≥400 IU/day, the results did not indicate any significant improvement in symptoms even after the third year of disease progression (Luthra et al., 2018). The randomized study determined that a high dose of VD, i.e., 10,000IU/day, improved the balance of PD patients, mainly in the younger population (Hiller et al., 2018). Moreover, such type of placebo-controlled moderate AD patients, when supplemented with 800 IU/day of VD for 12 months orally, showed significant improvement in cognitive function performance and decreased the level of Aβ related plasma markers, which determines the beneficial role of VD in AD patients (Jia et al., 2019). In a recent study on MS, supplementing placebo-controlled patients with 10,000IU to 40,000IU/day alone significantly reduced neuroinflammation-related abnormalities observed in MRI scans (Feige et al., 2020). Researchers found that the neurological symptoms of MS were significantly reduced when 40,000IU of VD were administered for five years, i.e., 1000IU/kg/day through a diet (Gandhi et al., 2021). Clinical studies on PD patients showed that 1200 IU of VD per day for 12 months had only a short-term effect when compared to

a placebo-controlled group, with serum concentrations of VD increasing to 19.2ng/ml after 12 months (Anwar et al., 2023). Furthermore, in the case of HD, it has been found that patients will have VD deficiency when they undergo treatment. However, there is no clinical evidence that VD is dose-dependent when treating HD patients.

1.4.6.1.2. The effect of different doses of VD in preclinical conditions:

At the pre-clinical stage, the protective effect of VD mainly depends on the type of condition and age, which leads to variations in the molecular changes. According to recent research on streptozocin-induced AD mice, 42IU/kg/day subcutaneous VD pre-supplementation for seven days and 42IU/kg/day oral VD post-supplementation for 21 days significantly improved cognitive function and rescued neurons from oxidative stress, neuroinflammation, synaptic dysfunction, and cholinergic dysfunction (Yamini et al., 2018). A study released in 2019 by Cui et al. concluded that intraperitoneal injections of 100 ng/kg VD for six weeks attenuated oxidative stress and inflammation in hypersensitive rats (Cui et al., 2019). There is an evidence that the anti-oxidative effect of VD on AD rats possessing $A\beta_{1-40}$ aggregates, when supplemented with 1µg/kg/day of it through i.p. for 14 days, will result in the survival of neurons, which is substantiated by the findings of histological examinations (S and Ss, 2020). As cited in 2022, Patel and Shah reported that oral administration of 2.5µg/kg/day and 5µg/kg/day of VD for 21 days to AD rats showed anti-oxidant, anti-inflammatory, and anticholinergic effects. In general, higher doses of VD have more favorable outcomes than lower doses, but the difference between the two doses is not very significant. Hence, it can be assumed that 2.5g/kg/day is a satisfactory dose for a person to demonstrate the beneficial effects (Patel and Shah, 2022). According to this, VD plays a valuable role depending on the dose and route of administration in different models of AD.

As well it has been found that in the case of PD, when the disease was induced with MPTP, the administration of 1g/kg/day of VD through intragastric gavage for 10 days had anti-inflammatory and antioxidant effects (Calvello et al., 2017). Furthermore, in 2018, it was determined that pre- and post-supplementation of 1µg/kg/day of VD for 7 days and 14 days, respectively, are beneficial. Based on the findings of the study, VD increases the expression of VDR in the 6-OHDA-induced mouse model of PD, whether it is administered

pre- or post-treatment (Lima et al., 2018). In 2022, Magdy et al. found that 1µg/kg/day of calcitriol administered for 8 days acted as an anti-oxidant and anti-apoptotic agent in PD mice induced by retenone (Magdy et al., 2022). A recent study found that VD supplementation of 1µg/kg/day for 21 days showed beneficial effects before and after exercise in PD rats. Based on the results of the study, the researchers concluded that VD has a protective effect by fighting inflammatory responses, and the cognitive function of rats does not differ depending on whether they have undergone exercise (da Costa et al., 2023). As a result, we can conclude that, in the case of PD, the effect of VD depends on the dose but not the time and route of administration.

Similarly, VD has beneficial effects on various other age-related neurological conditions, where the outcome depends on the dose and the route of administration, as shown in **Table 1**. A minimal amount of evidence indicates that VD supplements increase longevity in HD mice (Fort Molnár et al., 2016). As a result, it appears that the effectiveness of VD in treating various neurological disorders depends on the dose and route of administration.

Table 1. The beneficial effect of VD supplementation in different neurological diseases based on different doses both in *in-vitro* and *in-vivo* conditions

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
1.	Anti-inflammatory activity	Parkinson's disease	5 mg/kg of MPTP for four doses for every 8 hours – mouse model	1µg/kg/day of VD by intragastric gavage for 10 days	Decrease in the gene expression of pro-inflammatory markers (TNF-α, IL-1β). Increase in the gene expression of anti-inflammatory markers (IL-10, TGF-β) and protein expression of anti-inflammatory markers (CD163, CD204, CD206)	Calvello et al., 2017
			6-OHDA – rat model	Pre-supplementation of 1µg/kg/day for seven days, Post-supplementation of 1µg/kg/day for 14 days orally	Inhibiting protein expression of HSP40 and TLR4	Araújo de Lima et al., 2022
			6-OHDA - mouse model	30mg/kg/day of VD (i.p) for 21 days	Decrease in the gene expression of IL-1β	Bayo - Olugbami et al., 2022
		Aging	Spontaneously hypersensitive rats	100 ng/kg of calcitriol for 6 weeks orally	Decrease in gene expression of iL-1β, IL-6, IL-10	Cui et al., 2019

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
1.	Anti-inflammatory activity	Alzheimer's disease	2 mg/kg of scopolamine as a single dose intraperitoneally – rat model	2 doses of calcitriol were given orally for 21 days Doses: 2.5µg/kg/day and five µg/kg/day	Decrease in the concentration of IL-1β, TNF-α, interferon-γ, IL-6 and NF-κβ	Patel and Shah, 2022
		Ischemic stroke	Mice – middle cerebral artery occlusion	Pre-supplementation of 100ng/kg/day of calcitriol for 5 days	Decrease in the gene expression of TNF-α, IL-1β, IL-6, IL-21, IL-23a and TGF-β1	Evans et al., 2018
2.	Antioxidant activity	Parkinson's disease	SH-SY5Y treated with a solution of α-syn aggregates	(α-syn + VD) oligomers	Decrease in ROS generation	Zhang et al., 2022
			6-OHDA – rat model	Pre-supplementation of 1µg/kg/day for seven days, Post-supplementation of 1µg/kg/day for 14 days orally	Reduction in the levels of nitric oxide	Araújo de Lima et al., 2022
			5 mg/kg of MPTP for four doses for every 8 hours – mouse model	1µg/kg/day of VD by intragastric gavage for 10 days	Decrease in protein expression of iNOS	Calvello et al., 2017
			6-OHDA – mouse model	30mg/kg/day of VD (i.p) for 21 days	Decrease in the expression of MOAB, DDC	Bayo-Olugbemi et al., 2022

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
2.	Antioxidant activity	Parkinson's disease	6-OHDA – rat model	1 µg/kg/day of VD for 21 days orally	Decrease in nitrite and increase in GSH.	da Costa et al., 2023
			2.5mg/kg/day of rotenone for 4 weeks intraperitoneally – mouse model	1 µg/kg/day of calcitriol for 8 days	Increased the gene expression of *Sirt1*	Magdy et al., 2022
			SH-SY5Y cell line – exposed to MPP^+ for 24 hours	Pre-treated with calcitriol for 4 hours	Decrease in ROS production and increase in NAD^+ levels	Hu et al., 2021
		Alzheimer's disease	SH-SY5Y (Human neuroblastoma cell line) - 1µM of Aβ (25-35)	50nM of calcitriol (active form of VD) – 24 hrs after treatment with Aβ (25-35)	Decrease in intracellular ROS	Lin et al., 2020
			0.2µl of 5mg/ml of Aβ (1–40) peptides given directly to the dorsal right portion of the hippocampus – rat model	1µg/kg/day for 14 days	Increase in the activity of SOD	S and Ss, 2020

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
2.	Antioxidant activity	Alzheimer's disease	Primary neuronal culture from 1 week old rats - 1μM of Aβ (1-42)	1nM of VD treatment for 72 hours	Increase in levels of GSH, activity of GST, catalase, SOD	Alamro et al., 2020
			Spontaneously hypersensitive rats	100 ng/kg of calcitriol for 6 weeks orally	Decrease in gene expression of iNOS and protein expression of NOX2, NOX4. Decrease in the activity of NADPH oxidase, SOD, Cat.	Cui et al., 2019
			3 mg/kg of ICV-STZ injection	Pre-treatment of 42IU of VD for 7 days and post-treatment 42IU of VD for 21 days orally	Increase in the activity of SOD, Catalase, GSH	Yamini et al., 2018
			2 mg/kg of Scopolamine as a single dose intraperitoneally – rat model	2 doses of calcitriol were given orally for 21 days Doses: 2.5μg/kg/day and 5μg/kg/day	Decrease in the concentration of glutathione and activity of superoxide dismutase.	Patel and Shah, 2022
			0.8mg/kg of lipopolysaccharide for three weeks, which is once per week - rat model	VD: 1μg/kg twice a day for 4 weeks by i.p injection	Decrease in the gene expression of Keap1	Saad El-Din et al., 2020

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
2.	Antioxidant activity	Alzheimer's disease	SH-SY5Y neuroblastoma cell line –1μM of Aβ (25-35)	2 different concentrations of Calcitriol: 0.1nM and 10nM	Decrease in the level of intracellular ROS.	Lin et al., 2020
		Aging	5 months old rats, 12 months old rats, 24 months old rats	500IU/kg/day of VD for 5 weeks orally	Decrease in the level of MDA and increase in the activity of SOD	Khairy and Attia, 2021
			Human umbilical endothelial cells co-cultured with astrocytes	100nM of VD at different time points from 15 minutes to 1440 minutes	Decrease in ROS production, Decrease in the protein expression of SOD3	Molinari et al., 2019
			Primary cortical culture from 7 days old rats	4 different concentrations of Calcitriol: 0.25μg/ml, 0.5 μg/ml, 0.75 μg/ml and 1.0 μg/ml.	Decrease in lipid peroxidation and activity of catalase with Increase in the activity of GSH	AlJohri et al., 2019
		Huntington's disease	25 mg/kg of 3-NP for 3 doses for every 12 hours-mouse model	500IU/kg/day of VD (i.p) for 15 days	Decrease in the gene expression of GpX and Cat	Manjari et al., 2022

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
2.	Antioxidant activity	Depression	UCMS-rat model	3 doses of VD (i.p) for 4 weeks Doses: 100 IU/kg, 1000 IU/kg and 10,000IU/kg	Increase in the activity of Catalase, SOD.	Bakhtiari-Dovvombaygi et al., 2021b
3.	Neuroprotective role	Parkinson's disease	6-OHDA-rat model	1µg/kg/day of VD for 21 days orally	Increase in DA, TH, DAT	da Costa et al., 2023
			6-OHDA-rat model	Pre-supplementation of 1µg/kg/day for 7 days Post-supplementation of 1µg/kg/day for 14 days orally	Increase in the gene expression of BDNF and alteration of dopamine metabolism	Lima et al., 2018
			6-OHDA–mouse model	30µg/kg/day of VD (i.p) for 21 days	Increase in the gene expression of BDNF, DAT, and TH.	Bayo-Olugbami et al., 2022
			2.5mg/kg/day of rotenone for 4 weeks intraperitoneally – mouse model	1µg/kg/day of Calcitriol for 8 days	Increased the protein expression of TH	Magdy et al., 2022
			30mg/kg/day of MPTP for 7 days	2.5µg/kg/day of calcitriol for 7 days	Increase in the level of TH	Hu et al., 2021

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
3.	Neuroprotective role	Alzheimer's disease	SH-SY5Y (Human neuroblastoma cell line) - 1μM of Aβ (25-35)	50nM of Calcitriol (Active form of VD) – 24 hrs after treating with Aβ (25-35)	Increased the phosphorylation of PI3K, Akt, GSK-3β	Lin et al., 2020
			Colchicine dissolved in ACSF 15μg/rat (7.5in 5μl/site)	42 IU/kg subcutaneously for one week	Increase in the concentration of BDNF	AlJohri et al., 2019
			Primary neuronal culture from 1 week old rats - 1μM of Aβ (1-42)	1nM of VD and maintained for 72 hours	Increase in the concentration of NGF	Alamro et al., 2020
			0.2μl of 5mg/ml of Aβ (1–40) peptides given directly to the dorsal right portion of the hippocampus – rat model	1μg/kg/day for 14 days	Increase in the number of neurons on H&E staining	S and Ss, 2020
			APPswe/PS1ΔE9 mice	100ng/kg/day of calcitriol (i.p) for 6 weeks	Increase in the protein expression of VDR and decrease in the protein expression of p-ERK.	Bao et al., 2020

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
3.	Neuroprotective role	Alzheimer's disease	2 mg/kg of scopolamine as a single dose intraperitoneally – rat model	2 doses of calcitriol were given orally for 21 days Doses: 2.5µg/kg/day and 5µg/kg/day	Decrease in the concentration of Aβ and p-Tau	Patel and Shah, 2022
			0.8mg/kg of lipopolysaccharide for three weeks, which is once per week - rat model	1 µg/kg of VD twice a day for 4 weeks via i.p.	Decrease in the concentration of Aβ and p-Tau. Decrease in the protein expression of ERK1/2.	Saad El-Din et al., 2020b
			SH-SY5Y neuroblastoma cell line –1µM of Aβ (25-35)	2 different concentrations of calcitriol: 0.1nM and 10nM	Increase in the protein expression of VDR, GDNF. Decrease in the concentration of p-tau. Increased the phosphorylation of PI3K, Akt, GSK-3β	Lin et al., 2020
		Aging	5 months old rats, 12 months old rats, 24 months old rats	500IU/kg/day of VD for 5 weeks orally	Increase in the level of BDNF	Khairy and Attia, 2021
			Human umbilical endothelial cells co-cultured with astrocytes	100nM of VD at different time points from 15 minutes to 1440 minutes	Increase in ERK/MAPK activity and PI3K/Akt activity	Molinari et al., 2019

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
3.	Neuroprotective role	Huntington's disease	25 mg/kg of 3-NP for 3 doses for every 12 hours – mouse model	500IU/kg/day of VD (i.p) for 15 days	Increase in the gene expression of NGF, BDNF and protein expression of VDR	Manjari et al., 2022
		Depression	UCMS – rat model	3 doses of VD (i.p) for 4 weeks Doses: 100 IU/kg, 1000 IU/kg and 10,000IU/kg	Increase in the concentration of BDNF and decrease in the concentration of Aβ	Bakhtiari-Dovvombaygi et al., 2021b
		Stroke	CUMS – mouse model	4 doses of VD were given by ICV for 4 weeks Doses: 6µg/kg, 12.5µg/kg, 25µg/kg and 50µg/kg	Increase in the protein expression of VDR and BDNF	Xu and Liang, 2021
		Mild stress	CUMS – rat model	3 doses of VD were given by subcutaneous injection for 4 weeks Doses: 1.0mg/kg/day, 2.5mg/kg/day and 5mg/kg/day	Increase the protein expression of BDNF, NT-3, and NT-4.	Koshkina et al., 2019

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
4.	Anti-apoptotic activity	Aging	5 months old rats, 12 months old rats, 24 months old rats	500IU/kg/day of VD for 5 weeks orally	Decreased the activity of Caspase-3	Khairy and Attia, 2021
			Human umbilical endothelial cells co-cultured with astrocytes	100nM of VD at different time points from 15 minutes to 1440 minutes	Decrease in p53 activity, Decrease in the protein expression of cytochrome C	Molinari et al., 2019
			Primary cortical culture from 7 days old rats	4 different concentrations of Calcitriol: 0.25µg/ml,0.5 µg/ml, 0.75 µg/ml, and 1.0 µg/ml.	Increase in the number of viable cells	AlJohri et al., 2019
		Alzheimer's disease	Primary neuronal culture from 1 week old rats - 1µM of Aβ (1-42)	1nM of VD and maintained for 72 hours	Increase in the number of viable cells	Alamro et al., 2020
			SH-SY5Y (Human neuroblastoma cell line) - 1µM of Aβ (25-35)	50nM of Calcitriol (Active form of VD) – 24 hrs after treating with Aβ (25-35)	Decrease in the protein expression of activated caspase-3 with reduction in the percentage of apoptotic cells	Lin et al., 2020
			SH-SY5Y neuroblastoma cell line –1µM of Aβ (25-35)	2 different concentrations of calcitriol: 0.1nM and 10nM	Increase in cell viability. Decrease in the protein expression of caspase-3 and apoptotic cell death.	Lin et al., 2020

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
4.	Anti-apoptotic activity	Alzheimer's disease	3 mg/kg of ICV-STZ injection	Pre-treatment of 42IU of VD for 7 days and post-treatment 42IU of VD for 21 days orally	Increase in the activity of mitochondrial complexes and decrease in P65κB. Decrease in the number of dead neurons.	Yamini et al., 2018
			APPswe/PS1ΔE9 mice	100ng/kg/day of calcitriol by intraperitoneal injection for 6 weeks	Decrease in the percentage of apoptotic cells. Increase in the gene expression of Bcl-2. Decrease in the gene expression of Bax and caspase-3.	Bao et al., 2020
		Parkinson's disease	SH-SY5Y treated with a solution of α-syn aggregates	(α-syn + VD) oligomers	Decrease in cell death	Zhang et al., 2022
			6-OHDA – mouse model	30mg/kg/day of VD (i.p) for 21 days	Decrease in the gene expression of BAX	Bayo-Olugbami et al., 2022
			2.5mg/kg/day of rotenone for 4 weeks intraperitoneally – mouse model	1µg/kg/day of calcitriol for 8 days by subcutaneous injection	Increased the expression of LC3 and decrease in the protein expression of P62, NF-κB	Magdy et al., 2022

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
5.	Anticholinesterase activity	Aging	5 months old rats, 12 months old rats, 24 months old rats	500IU/kg/day of VD for 5 weeks orally	Increase in the activity of AChE	Khairy and Attia, 2021
		Alzheimer's disease	Intracerebroventricular injection of 3 mg/kg of streptozotocin – rat model	3 different doses of VD, i.e., 12.5µg/kg, 42µg/kg, and 125µg/kg orally for 21 days	Decrease in AChE activity	Rodrigues et al., 2019
			3 mg/kg of ICV-STZ injection	Pre-treatment of 42IU of VD for 7 days and post-treatment 42IU of VD for 21 days by oral dose orally	Decrease in activity of AChE	Yamini et al., 2018
			2 mg/kg of Scopolamine as a single dose intraperitoneally – rat model	2 doses of calcitriol were given orally for 21 days Doses: 2.5µg/kg/day and 5µg/kg/day	Decrease in the level of AChE	Patel and Shah, 2022
6.	Immunomodulatory effect	Parkinson's disease	6-OHDA – mouse model	30mg/kg/day of VD (i.p) for 21 days	Decrease in the gene expression of CD11b	Bayo-Olugbemi et al., 2022
		Alzheimer's disease	Spontaneously hypersensitive rats	100 ng/kg of calcitriol for 6 weeks orally	Decrease in gene expression of TNFα, CD86, CD206, Arg1	Cui et al., 2019

S. No.	Mode of action	Disease type	Model	Dose of VD	Mechanism of action	Ref.
6.	Immunomodulatory effect	Alzheimer's disease	0.8mg/kg of lipopolysaccharide for three weeks, which is once per week - rat model	1 µg/kg of VD twice a day for four weeks through i.p.	Decrease in the protein expression of MAPK P38	Saad El-Din et al., 2020

1.4.6.1.3. The cellular effect of VD in various neurological conditions:

A thorough investigation of the mechanisms of action of calcitriol was undertaken using *in-vitro* models for different neurological conditions. A study on primary cortical neurons from 7-day-old rats treated with VD at four different doses, i.e., 0.25µg/ml, 0.5µg/ml, 0.75µg/ml, and 1µg/ml, showed an improved survival rate of existing cells after 72 hours. It was concluded that the protective role of VD led to a reduction in oxidative stress, decreased catalase activity and an increase in glutathione transferase activity (AlJohri et al., 2019). Another study conducted in the SH-SY5Y neuroblastoma cell line, induced with 1µM of Aβ (25-35), indicated dose-dependent effects of calcitriol (0.1nm or 10nm) in AD. The beneficial effects of VD was shown to occur by increase in the gene expression of VDR, decrease in caspase-3 gene expression and an enhancement in the AKt kinases signaling pathway (Lin et al., 2020). A study by Alamro and colleagues demonstrated the beneficial effect of VD when primary neuronal cultures from one-week-old rats were treated with 1µM of Aβ (1-42). The treatment of cells with 1nM of calcitriol for 72 hours significantly reduced the activity of antioxidants like SOD and catalases and increased the gene expression of NGF, demonstrating the survival of neurons (Alamro et al., 2020).

Similarly, VD supplementation has been shown to protect against PD through various signaling pathways. According to one study, when SH-SY5Y neuroblastoma cells were exposed to MPP+, they developed PD-like features. The study showed that pre-treatment with calcitriol for four hours reduced ROS production in cells and increased VDR expression, indicating that VD has a beneficial effect on oxidative stress (Hu et al., 2021). Later, a study on SH-SY5Y neuroblastoma with α-syn aggregates, when supplemented with calcitriol, showed its anti-oxidant effect by decreasing the ROS production, thereby decreasing the number of apoptotic cells (Y. Zhang et al., 2022).

Similarly, VD also has a protective role against apoptotic cell death, oxidative stress, and an increase in the expression of neurotrophins in the central nervous system, as shown in **Table 1**. Based on the findings above, it is evident that VD has a beneficial effect on a wide range of molecular markers to evaluate the mechanism of action in the brain through *in-vitro* studies.

1.4.6.2. Effect of VD deficiency in HD:

Huntington's disease (HD) is one of the age-related neurodegenerative disorders with high prevalence found mostly in Caucasians. A lack of VD is among the most common health problems among people suffering from age-related neurological disorders like HD. From the initial clinical study, it may be considered that the risk of fractures is higher in patients who suffer from HD (Grimbergen et al., 2008). According to a study by Godman et al., there is an increased risk of VD deficiency, which may occur before the outbreak of HD (Goodman and Barker, 2011). Based on the clinical study conducted by Chel et al., about 89% of patients with HD have VD insufficiency, with an average serum concentration of calcidiol at 33 nmol/L (Chel et al., 2013). Therefore, it can be considered that VD deficiency and insufficiency are highly observed in patients with HD.

1.5. Huntington's disease (HD):

1.5.1. Historical significance of HD:

Huntington's disease (HD) was diagnosed in 1842 as Huntington chorea, but by 1872, it had been renamed Huntington's disease by George Huntington himself (Roos, 2010). HD is an autosomal dominant disease mainly characterized by behavioral, motor, cognitive, and neuropsychiatric symptoms (Gatto and Weissmann, 2022). There is an average age of 35 to 44 years when the signs of the disease will appear, while the average survival time after the onset of the symptoms will be 15 to 18 years (Caron et al., 2020). As of 2010, there are 17,2 cases per 100,000 of HD in the Caucasian population, and it has been estimated that there are more than 0.2 million people in India who are at risk because of HD (Raju and Kukkle, 2021; Shaw et al., 2022).

The disease is caused by an autosomal dominant inherited CAG repeat located in the huntingtin gene (*Htt*) on the 4th chromosome, which causes its manifestation. In the 1st exon of the mRNA, repeats of the CAG motif will be found at the N-terminus in a polyglutamate expansion of more than 40 repeats (Capiluppi et al., 2020). There is an inverse correlation between the number of CAG repeats and the age of onset of the disease (Pringsheim et al., 2012). In most cases, the onset of symptoms occurs after 45 years of age, but if the symptoms appear before 31, this is considered a juvenile onset of the disease

(Machiela et al., 2020). The diagnostic criteria of the disease are mainly carried out by MRI imaging and genetic analysis based on the comprehensive family medical history brain imaging, and genetic testing (Caron et al., 2020).

1.5.2. Prevalence of HD:

HD is diagnosed clinically through a combination of family history, personal history, and neurological, psychiatric, and genetic testing (Medina et al., 2022; Roos, 2010). A classification scheme divides HD into presymptomatic, prodromal, and manifest forms (Ross et al., 2019). Presymptomatic HD patients have CAG repeats without any symptoms associated with HD (Wheeler et al., 2007). Individuals with prodromal HD may have CAG repeats as well as motor abnormalities and apparent cognitive symptoms (Medina et al., 2022). Manifest HD includes 90% of individuals with motor abnormalities and cognitive changes, whereas 99% have no cognitive impairment or motor abnormalities (Ross et al., 2019).

According to the initial pooled evidence of the clinical studies, that prevalence is 0.38 cases per 100,000, with a global prevalence of 2.71 cases per 100,000 (Pringsheim et al., 2012). The increase in the prevalence of HD may be attributed to the earlier diagnosis and medical treatment of the disease (Rawlins et al., 2016). Moreover, de novo mutations are estimated to account for 7.1% of all new cases, which may contribute to the rising incidences of this disease (Kay et al., 2018). A lower incidence of cases is observed in Asian countries than in Europe, North America, and Australia (Pringsheim et al., 2012). Caucasian populations have an overall prevalence of 8.2 to 9.0 cases per 100,000, whereas the overall prevalence in Asia is 0.99 cases per 100,000 people (Medina et al., 2022).

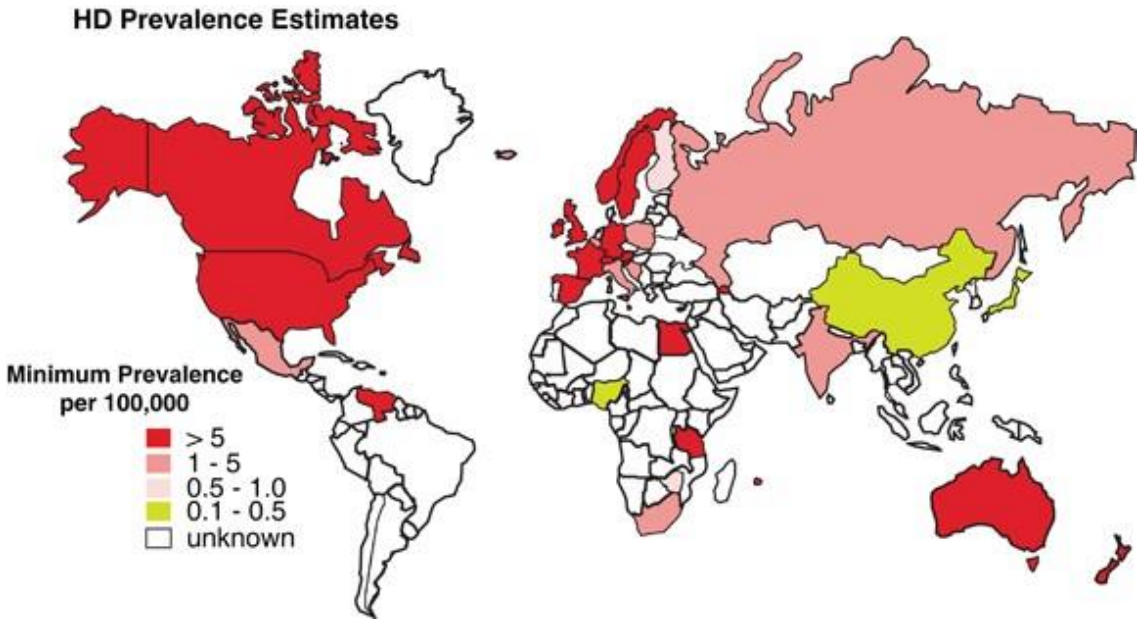

Fig.8. Prevalence of HD: is increasing daily worldwide, increasing in different regions as a growing public health problem that might change with time. (Image source: Warby et al., 2011)

It is worth noting that there is very little evidence available from India, where a prevalence of 1.75 cases per 100,000 people is reported, which is mainly seen in Indian immigrants from the UK (Shiwach and Lindenbaum, 1990). The first well-documented case was from India, especially in the northern part of the country, with 35 sporadic cases over 11 years (Khosla and Arora, 1973). Several years later, in 2001, clinicians from Nimhans found 26 subjects exhibiting symptoms that were native to South India and that had onsets of the disease between the ages of 6 and 66 (Murgod et al., 2001). In this group of patients, about 88.5% of the patients have initial motor symptoms, followed by 11% who have behavioral problems (Murgod et al., 2001). The same institute also found psychiatric comorbidity in 144 South Indian patients whose mean death age was 53 and whose illness duration was 7 years (Ratna et al., 2022). In recent research from Eastern India, 75 HD subjects with a mean age of onset of 37 years and 5% of patients with juvenile onset of the disease were found (Hussain et al., 2020). A recent study by Nimhans observed 3 to 5 HD cases for every 100,000 people, which gives a total of 40000 to 70,000 HD cases in the total population. As a result of all this evidence, it can be concluded that HD is well recognized in all parts of the country.

1.5.3. Pathogenesis of HD:

HD is an autosomal dominant neurodegenerative disorder characterized by movement disorders and cognitive decline (Roos, 2010). Clinically, HD is mainly characterized by the shrinkage of the brain with the degeneration of the striatum and cortex, with a specific loss of medium spiny neurons (MSNs; Blumenstock and Dudanova, 2020; Le Cann et al., 2021; Reiner et al., 1988). However, on disease progression, other brain regions like globus pallidus (GP), thalamus, hypothalamus, subthalamic nucleus, substantia nigra (SN), and cerebellum may also get affected (Heinsen et al., 1996; Hp et al., 1990; Kassubek et al., 2004; Petersén et al., 2002). HD is a progressive neurodegenerative disorder caused by the mutation in the huntingtin (*Htt*) gene, a ubiquitously expressed protein of 350KDa (Jimenez-Sanchez et al. 2017). The *Htt* gene is widely expressed in humans and rodents, with the highest levels of expression in neurons of the central nervous system, mainly striatal and corticostriatal neurons (DiFiglia et al., 1995; Fusco et al., 1999). Huntingtin is a complex protein containing a polyglutamine tract encoded by trinucleotide repeats uninterrupted across the first exon (Iennaco et al., 2022; Jimenez-Sanchez et al., 2017). HD patients carry expansions of 36 or more CAG repeats compared to wild-type alleles, which contain 35 CAG repeats (Warby et al., 2009; Wheeler et al., 2007). It has been shown that a strong correlation exists between the number of CAG repeats and the age of onset of symptoms, with greater CAG repeat expansions generally associated with earlier onset ages of symptoms (Langbehn, 2022; Swami et al., 2009). Understanding the structure and function of wild-type Htt genes and proteins is essential before studying HD pathology.

1.5.3.1. Structure and function of wild-type Huntingtin gene and protein:

A wild-type Huntingtin protein (350KDa) consists of polyglutamine sequences at the NH2 terminus as well as multiple consensus sequences known as HEAT (huntingtin, elongation factor 3, protein phosphatase 2A, and TOR1 [target of rapamycin 1]) repeats that play a crucial role in protein–protein interactions (Pryor et al., 2014; Ramazzotti et al., 2012; Sap et al., 2021; Sapp et al., 2020; Takano and Gusella, 2002). The motifs mainly involve intracellular trafficking (Chen et al., 2023). It is a cytoplasmic protein that has

partial nuclear localization and colocalizes with a range of cell organelles, such as the nucleus, the endoplasmic reticulum, the Golgi complex, and the endosomes (Schulte and Littleton, 2011). In addition to this, huntingtin possesses a nuclear export sequence, which can be found near its COOH terminus (McClory et al., 2018). Moreover, the N-terminal sequence in huntingtin interacts with Tpr, a nuclear export protein involved in nuclear export (Cornett et al., 2005).

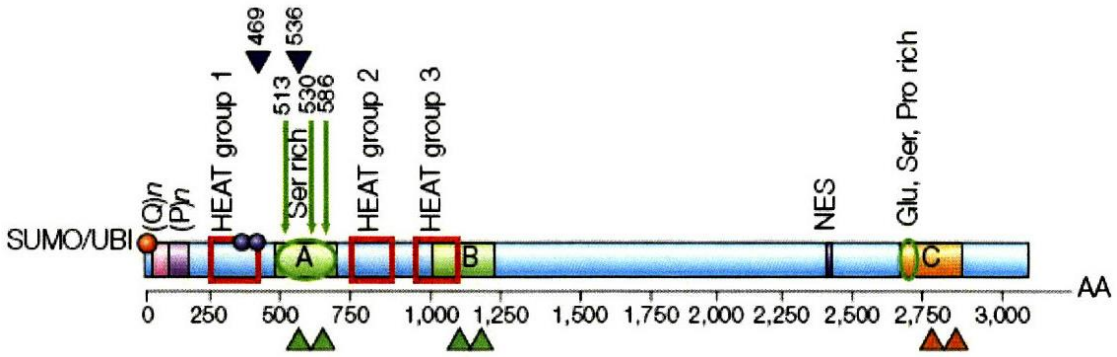

Fig.9. Diagram showing Huntingtin's amino acid structure. (Q)n indicates polyglutamine regions and P(n) indicates polyproline regions. There are 37 HEAT repeat domains clustered into three main groups (red boxes). In the circles, posttranslational modifications are indicated (sumoylation/ubiquitination at the red circles, phosphorylation at the blue circles). An arrowhead indicates the site of caspase cleavage and a triangle indicates calpain cleavage. NES stands for nuclear export signal sequence. (Image source: Paine, 2015)

Since the Huntingtin gene is responsible for HD, the wild-type Huntingtin protein has certain functions (Schulte and Littleton, 2011). It has been determined that it plays a significant role in embryonic development, as it stimulates neurogenesis, maintains neural stem cells, and regulates neuroendothelial interactions (Barron et al., 2021; Conforti et al., 2013; Jeong et al., 2006; Sari, 2011). It acts as a scaffolding protein that interacts with β-tubulin and binds to the microtubules, thereby maintaining the cytoskeleton (Rui et al., 2015; Schulte and Littleton, 2011; Tousley et al., 2019). The protein also plays a role in transcription regulation, mainly for the BDNF (Bathina and Das, 2015). In recent research, huntingtin has been shown to interact with methyl-CpG-binding protein 2, thereby modulating BDNF transcription (McFarland et al., 2014). Furthermore, it regulates synaptic function by interacting with synaptic vesicles at the pre-synaptic terminal and

PSD95 at the post-synaptic terminal (Barron et al., 2021; Chen et al., 2021; Wennagel et al., 2022). A recent study found that huntingtin is essential in forming the excitatory synapses between cortical and striatal areas (Blumenstock and Dudanova, 2020). In light of the above evidence, it can be concluded that Htt protein plays a vital role in the normal functioning of neurons.

1.5.3.2. Neuropathology of HD:

The primary characteristic of HD is the loss of medium spiny neurons, primarily in the striatum, followed by the cortex (Blumenstock and Dudanova, 2020; Ehrlich, 2012). In the striatum, MSNs constitute nearly 90-95% of the neurons that conduct neurotransmission through gamma amino butyric acid (GABA) and glutamate (André et al., 2010; Arama et al., 2015; Garret et al., 2018; Lee et al., 2016). The striatum receives input from specific thalamic nuclei and neocortical areas (Lanciego et al., 2012). Furthermore, the striatum contains several modulatory components, including dopamine (DA), which is released from the subsubstantia nigra pars compacta (SNc) of the brain (Yamada et al., 2016; Zhai et al., 2019). Striatal output is primarily divided into two populations of MSNs with distinct projections and DA receptors that involve direct and indirect pathways (Andre et al., 2011). The direct pathway is composed of neurons expressing D1 DA receptors as well as substance P (Sp) that send projections to substantia nigra pars reticulata (SNr) and globus palladius interna (GPi; Gerfen, 2023; Wall et al., 2013). An indirect pathway is mediated by neurons expressing predominantly D2 receptors and extending to the globus palladius externa (GPe; Cazorla et al., 2015; Gerfen, 2023). Through the balance between direct and indirect pathways through the excitatory and inhibitory signals conveyed by glutamate and GABA, respectively, the movement of the body is maintained (Cazorla et al., 2015; Lanciego et al., 2012).

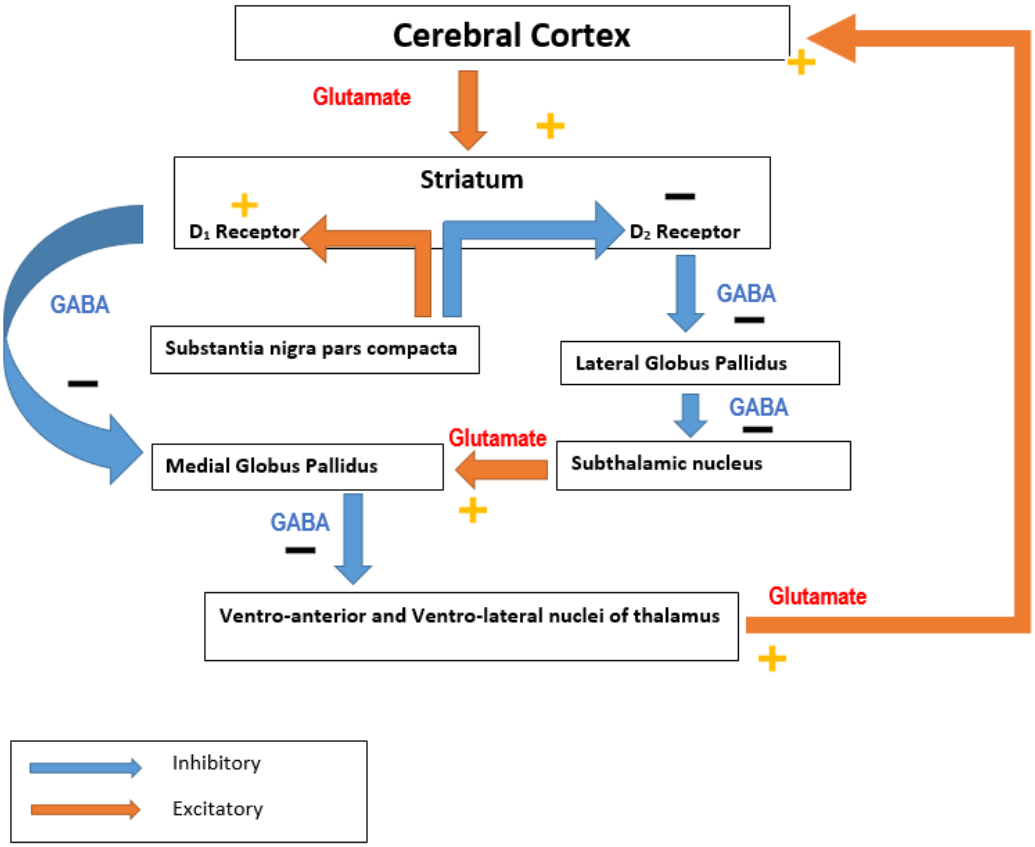

Fig.10. Direct and indirect pathways of Basal Ganglia in initiating motor activity. In the direct pathway, cerebral cortical input to the striatum causes activation of inhibitory neurons in the striatum which then causes an increased inhibitory output to the globus pallidus internal [GPi]. There is a decreased inhibitory output from GPi to ventral anterior [VA] and ventral lateral [VL] nuclei of the thalamus which then projects via excitatory pathways into the premotor cortex. The direct pathway is involved in regulating tonic excitation in the premotor cortex which is an area involved in planning and initiating movement. The indirect pathway is inhibitory to movement when excitatory projection from cerebral cortex facilitates inhibitory projection neurons in globus pallidus external [GPe]. These then inhibit tonic inhibitory output neurons which decreases tonic inhibition of subthalamic nucleus [STN] resulting in increased excitatory output to GPi. Excitatory input to GPi increases inhibitory output from GPi to thalamus which then decreases excitatory feedback to cerebral cortex leading to inhibition of motor activity. Dopamine promotes action of direct pathway while suppressing the activity of indirect pathway. (Image source: Roshan et al., 2016)

As the disease progresses by there is initially loss of medium spiny neurons of indirect pathway. As there is progressive degeneration of these neurons takes place, the excess glutamatergic and dopimenergic signals that would have gone to the indert pathway, will be funneled to direct pathway. As the disease progresses by the neurons which are involved in the direct pathway will also undergo gradually at the later stages of the disease. It is presumed that if the neurons associated with the indirect pathway undergo degeneration, a change or loss of input to the GPe will lead to an imbalance in the output circuit of the basal ganglia, followed by dystonia-like symptoms at the later stages, which are the result of the loss of neurons expressing D2 receptors (Andre et al., 2011; Galvan et al., 2012; Garret et al., 2018; Nelson and Kreitzer, 2014; Rikani et al., 2014). According to these observations, HD is characterized by hyperkinesia and hypokinesia due to the loss of MSNs.

1.5.3.3. Molecular mechanisms involved in neuronal atrophy in HD:

It is well known that HD is mainly caused by the genetic origin of the disease due to the expansion of CAG repeats at the N-terminal end of the *Htt* gene, which is located on the 1st exon of the 4^{th} chromosome (Gil-Mohapel, 2012; Möncke-Buchner et al., 2002). When the CAG repeats exceed 36, symptoms of the disease may arise (Wheeler et al., 2007). One of the hallmarks of HD on a neuropathological level is the formation of protein aggregates originating from the post-translational modification of *mHtt* (Cisbani and Cicchetti, 2012; Jarosińska and Rüdiger, 2021). The accumulation of polyglutamate aggregates may result in neuronal loss due to their interaction with up to 800 proteins (Jarosińska and Rüdiger, 2021). Their functions may include RNA binding, neurogenesis, transcription, translation, cytoskeletal organization, mitochondrial function, vesicular transport, and synaptic transmission (Barron et al., 2021; Eshraghi et al., 2021; Heinz et al., 2021; Nguyen et al., 2013; Tousley et al., 2019). The toxicity in the neurons with *mHtt* occurs due to the gain of function by the expanded and mutated expression of polyglutamine (Sari, 2011). In turn, mutant protein aggregates will become more prone to proteolysis, and toxic polyglutamate fragments will interfere with other proteins, causing neuronal dysfunction and selective neuronal death (Caron et al., 2020; Sari, 2011). An association exists between polyglutamine length and disease threshold (Lieberman et al.,

2019). Based on previous studies, these protein aggregates disrupt axonal transport and autophagy (Jarosińska and Rüdiger, 2021; Kim and Kim, 2014). The *mHtt* may also have toxic effects. Previous studies have shown that nuclear localization of *mHtt* increases cellular toxicity (Bae et al., 2006). The interference with neurotrophin transport may result in neuronal dysfunction, leading to neuronal death (Gatto et al., 2020). Aggregates of mHtt may also be involved in proteolytic cleavage (Gray et al., 2008). A study has shown that huntingtin can undergo proteolytic cleavage, resulting in the generation of toxic fragments transported to the nucleus (Gray et al., 2008). The toxic fragment of mHtt or the entire length of mHtt can interfere with BDNF transport in the nucleus and inhibit its functioning (Gatto et al., 2020). When mHtt is present, proteins may misfold, leading to protein aggregation and ubiquitination (Ross and Tabrizi, 2011; Takahashi et al., 2008).

Growing evidence suggests that transcriptional dysregulation is the major pathogenic mechanism of HD (Riley and Orr, 2006). Transcription factors implicated in HD pathophysiology include repressor element-1 silencing transcription factor (REST), CREB, NF-κB, and peroxisome proliferator-activated receptor gamma coactivator-1 alpha (PGC-1α) (Chaturvedi et al., 2009; Glass et al., 2000; Hsiao et al., 2013; Zuccato and Cattaneo, 2007). REST is the major response element for BDNF promotor II and acts as a regulator for the expression of BDNF (Zuccato and Cattaneo, 2007). When mHtt dissociates from REST, it facilitates its translocation to the nucleus, suppressing gene transcription and decreasing BDNF expression (Zuccato and Cattaneo, 2007). Similarly, CREB binds to DNA sequences called cAMP response elements (CRE) and increases or decreases downstream gene expression (Finkbeiner, 2000). CREB supports cell survival by enhancing the expression of cell-protective proteins, such as BDNF and Bcl-2, whereas the activity of CREB is downregulated in HD patients (Finkbeiner, 2000). NF-κB is responsible for the production of cytokines and is involved in the regulation of immunity, synaptic plasticity, and cell survival (Kaltschmidt and Kaltschmidt, 2015, 2009; Mattson and Meffert, 2006; Shih et al., 2015). One study found that enhanced NF-κB activation increased astrocyte inflammation and HD pathophysiology, suggesting an increased rate of inflammation and apoptotic cell death in HD (Hsiao et al., 2013; Marcora and Kennedy, 2010).

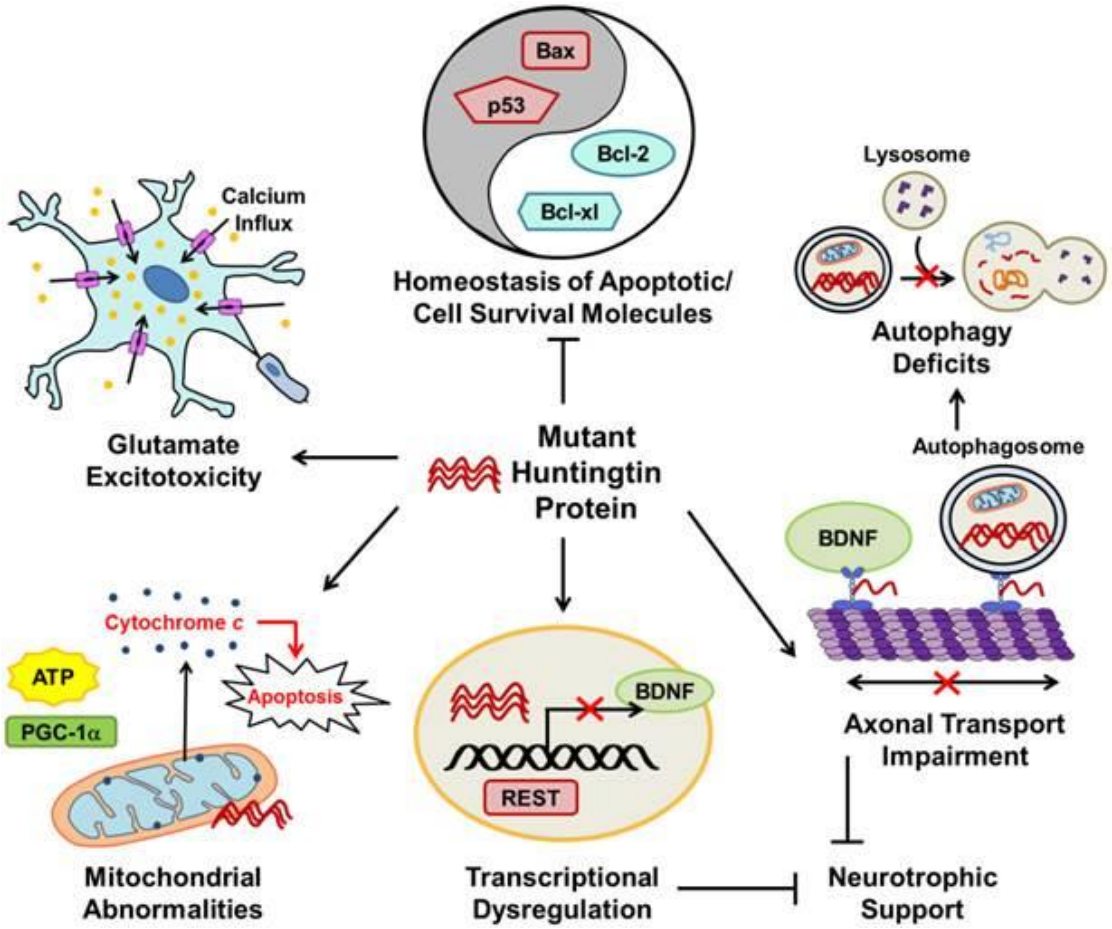

Fig.11. The diagrammatic representation of major physiological pathways in HD. Several physiological processes are disrupted by mutant huntingtin protein (mHtt), including apoptosis, autophagy, axonal transport, transcription, neurotrophins, mitochondrial function, and excitotoxicity. A pointed arrow indicates an increase in physiological events, while a blocked arrow indicates an inhibition. (Image source: Scheuing et al, 2014)

One of the major pathologies in HD observed is weight loss, increased calorie consumption, and metabolic dysfunction (Handley et al., 2016; Marder et al., 2009; Ogilvie et al., 2021). These metabolic abnormalities may occur due to the decreased activity of oxidative phosphorylation and decreased ATP production (Mochel and Haller, 2011; Powers et al., 2007). It is well known that Peroxisome proliferator-activated receptor-gamma coactivator (PGC-1α) is the key regulator for mitochondrial function, and the decreased expression of PGC-1α is seen in the patients with HD depicting the disbalance in the autophagy-lysosome pathway (Johri et al., 2013; Yang and Zhang, 2023). It is also well known that the later stages of HD may lead to other age-related neurodegenerative

diseases like AD and PD due to the interaction of mHtt aggregates with Tau proteins and α-synuclein (Jarosińska and Rüdiger, 2021; Masnata et al., 2020). All these evidences determine that dysregulation in the confirmation and processing of mHtt may represent the core molecular pathology of HD, leading to neuronal dysfunction and neuronal atrophy.

1.5.4. Different animal models of HD:

Animal models are needed to understand the pathogenesis of HD, elucidate brain regions involved in structural and functional decline, and evaluate potential therapeutic interventions. A reliable model of HD should be able to mimic the neuropathology and symptoms of HD. Selecting the most appropriate animal model is crucial, primarily a rodent model (Ramaswamy et al., 2007). Many invertebrate models are used to study HD pathology and therapeutic strategies, including *C. elegans* and *Drosophila melanogaster* (Krench and Littleton, 2013; Machiela et al., 2016). Most HD animal models can be categorized as genetic or non-genetic.

1.5.4.1. Genetic models of HD:

The discovery that *mHtt* is the cause of the disease prompted genetic models to be investigated to answer basic biological questions about it and find potential treatments. A genetic model can also be classified into two categories, i.e., transgenic and knock-in.

1.5.4.1.1. Transgenic models of HD:

The generation of transgenic mice involves the introduction of HD mutation in the mouse germline. It could, therefore, be predicted to generate a mouse model of HD despite the presence of two copies of the huntingtin gene. Several transgenic models are available for studying HD pathology, as shown in the following **Table 2**:

Table 2: List of transgenic animal models to study the pathology of HD

Animal model	Species	Construct	No. of CAG repeats	Cells effected	Symptoms	Ref.
R6/2	Mouse	First 90 amino acids of human Htt are randomly inserted into mouse genome	144	Significant neuronal loss in striatum	Chorea like movements, seizures, weight loss, motor dysfunction, decrease in memory	Dodds et al., 2014; Ellrichmann et al., 2017
R6/1	Mouse	First 90 amino acids of human Htt are randomly inserted into mouse genome	116	Decrease in striatal volume and presence of cellular inclusions	Decrease in body weight, gait abnormalities, decrease in anxiety	Gatto and Weissmann, 2022; Naver et al., 2003
N171-82Q	Mouse	First 171 amino acids of yeast artificial chromosome of human Htt are randomly inserted into mouse genome	82	Neuronal loss in striatum. 20% of cell shrinkage. Presence of cellular inclusions in striatum, hippocampus and cortex	Motor dysfunction, Clasping behavior, weight loss, decrease in working memory	Ferrante, 2009; Fort Molnár et al., 2016
YAC	Mouse	Yeast artificial chromosome expressing entire human Htt protein	72, 128	Majority of loss in lateral striatum	Hyperkinesia, gait abnormalities, ataxi	Figiel et al., 2012; Slow et al., 2003
Transgenic rat	Rat	1962 base pairs from the N-terminal end of rat *Htt* gene	51 human derived repeats	Enlargement of lateral ventricles and presence of cellular inclusions throughout the brain	Motor dysfunction, gait abnormalities, dyskinesia, decline in working memory	von Hörsten et al., 2003

1.5.4.1.2. Knock-in models of HD:

A knock-in mouse model of HD is considered the most accurate genetically. To create these animal models, a mutant human copy of the *Htt* gene with expanded CAG repeats is replaced with the wild-type portion. As a result, these animals contain two copies of the *Htt* gene, the wild-type and mutant type. Here are some of the most commonly used HD knock-in models:

Table 3: List of knock-in models of HD

Model	Species	Construct	No. of CAG repeats	Cells affected	Symptoms	Ref.
HdhQ92	Mouse	Replacing exon 1 of mouse Htt with an exon 1 of mutant human Htt	92	No striatal degeneration	No symptoms	Brooks et al., 2012; Wheeler et al., 2000
HdhQ111	Mouse	Replacing exon 1 of mouse Htt with an exon 1 of mutant human Htt	111	No striatal degeneration	Gait abnormalities	Wheeler et al., 2000; Yhnell et al., 2016
CAG 140	Mouse	Inserting CAG repeats into mouse Htt gene	140	Nuclear inclusions in striatum, cortex, hippocampus and cerebellum	Increase in anxiety and decrease in stride length	Hickey et al., 2012; Kaye et al., 2021
CAG150	Mouse	Inserting CAG repeats into mouse Htt gene	150	Increase in striatal gliosis and myelin breakdown	Motor dysfunction, clasping behavior, hypoactivity, gait disturbances	Brooks et al., 2012b; Heng et al., 2007

There are some drawbacks to using genetic models to study mutant huntingtin protein (Ramaswamy et al., 2007). These are as follows:

1. There is a possibility that the mutated gene inserted into the genetic model may interfere with the normal function of other genes that are not related to HD.

2. *Htt* expression is controlled spatially and temporally by an artificial promoter, whose control differs from the endogenous promoter's.

3. The exact behavioral deficits seen in HD patients may or may not be apparent and usually take much longer to manifest.

Considering all these factors, one can propose a non-genetic model of HD to study its pathology and therapeutic strategies.

1.5.4.2. Non-genetic model of HD:

A non-genetic model is generally used to test the therapeutic effects of drugs that are either in use or to be planned for the use in human clinical trials (Maze et al., 2015; Upadhyay et al., 2023). Along with this a non-genetic model have also been effective for testing neurotrophic factors, oxidative stress, inflammation, and mitochondrial dysfuction which are the main pathogenic conditions of HD (Mundo et al., 2013; Upadhyay et al., 2023). This determines that the neuroprotective therapies in a non-genetic model may be useful for understanding the benefits of treating patients who are diagonized with HD either by onset of neuronal degeneration or by behavioral symptoms (Ramaswamy et al., 2007; Túnez et al., 2010). These models can induce cell death by excitotoxicity or altering mitochondrial metabolism. To produce non-genetic models of HD, some neurotoxins can be used, as shown below:

Table 4: List of neurotoxins to be used to generate the non-genetic model of HD to study the pathology and therapeutic strategies of different drugs

Type of neurotoxin	Species	Mode of administration	Cells affected	Mode of action	Symptoms	Ref.
Kainic acid (KA)	Rats	Intrastriatal injections	Striatal cholinergic and GABAergic neurons	Excitotoxicity due to the excessive stimulation of kainite receptors	Impaired learning and memory, locomotor impairment	Coyle, 1979; Coyle et al., 1983
Malonic acid (MA)	Rats	Intrastriatal injections	Striatum	Reversible inhibitor of succinate dehydrogenase leading to mitochondrial dysfunction, excitotoxicity and generation of ractive oxygen species	Decreased locomotion, grip strength and spatial memory	Kalonia et al., 2010; Kumar et al., 2013
Quinolenic acid (QA)	Rats, mouse and non-human primates	Intrastriatal or intraperitoneal injections	Striatal depletion and selective loss of GABAergic neurons	Excitotoxicity, inflammation, oxidative metabolism	Hyperkinesia, dyskinesia and memory deficits	Sanberg et al., 1989

Type of neurotoxin	Species	Mode of administration	Cells affected	Mode of action	Symptoms	Ref.
3-nitropropionic acid (3-NP)	Rats, mouse and non-human primates	Intraperitoneal or intrastriatal injections	GABAergic medium spiny neurons, lateral striatum and cortex	Mitochondrial dysfunction by irreversible inhibition of succinate dehydrogenase	Hyperkinesia, hypokinesia, dystonia, dyskinesia, impaired spatial memory	Borlongan et al., 1997; Túnez et al., 2010

According to the **Table 4,** 3-NP model is the widely used model to study the neuropathology of HD due the following reasons:

1. Neurotoxicity can be studied with this method because it results in massive cell death in a short period of time.

2. One can study the therapeutic potential of different drugs on neuroprotection and neuro restoration by using this model.

3. The model is helpful in analyzing synergetic effects caused by mitochondrial alterations and energy depletion.

4. The model is most suitable to study the dose dependent activity of different drugs at different stages of disease.

Hence, 3-NP induced animals are considered to be the best model for studying the therapeutic effects of different neutraceuticals like VD (Túnez et al., 2010).

1.5.5. 3-Nitropropionic acid induced mouse model of HD:

3-nitropropionic acid (3-NP) is a natural toxin synthesized from fungi and plants which has tendency to cross the blood-brain barrier (Silva-Palacios et al., 2017). The model can mimic hyperkinetic and hypokinetic symptoms of HD depending on the time and dose of administration (Borlongan et al., 1997; Storgaard et al., 2000). It acts as an irreversible inhibitor of tricarboxylic acid cycle and respiratory chain enzyme succinate dehydrogenase (SDH), the main constituent of mitochondrial respiratory chain complex II (Herrera-Mundo and Sitges, 2013; Silva-Palacios et al., 2017). This permanent inhibition leads to deficits in ATP synthesis in the mitochondrial membrane, increasing the lactate concentration and causing hypoxia in cells followed by neuronal death primarily in the striatal region, which results in dystonia (Maciel et al., 2004; Nasr et al., 2003; Schulz et al., 1996). Apoptosis is also caused by ATP synthesis being disrupted by 3-NP induction, which affects several biochemical pathways. As a result of the disruption of the mitochondrial membrane, cytochrome-c is more readily expressed, resulting in the activation of caspase-9, which further stimulates caspase-3 by acting on pro-caspases and affecting neuronal integrity (C et al., 2014).

In addition, 3-NP induced mitochondrial abnormalities affect the oxidative stress, thereby increasing the level of reactive oxygen species (ROS) associated with HD (C et al., 2014). It has been shown that 3-NP increases free radical and fatty acid levels in rodent models (Binienda and Kim, 1997). Moreover, 3-NP induction reduces the activity of antioxidant enzymes like SOD, GpX, and Cat, thereby further promoting ROS production (Kumar et al., 2007, 2006). In addition, excess generation of ROS damages mitochondrial DNA and reduces the expression of anti-apoptotic markers like Bcl-2, leading to motor abnormalities and cognitive deficits (Colle et al., 2012; Mandavilli et al., 2005).

Neuronal excitotoxicity can occur in the brain due to 3-NP altering Ca^{2+} homeostasis and over activating NMDA receptors, leading to neuronal degeneration, a contributing factor to HD (Chidambaram et al., 2017). The calcium/calmodulin pathway promotes neuronal excitotoxicity when NMDA receptors are overactivated, resulting in an excess release of nitric oxide synthase (nNOS) (Calabresi et al., 2001). As a result of overactivation of these NMDA receptors and increased Ca^{2+} influx, caspases are activated, which in turn lead to striatal lesions, which constitute a major sign of disease progression (Browne and Beal, 2006; Calabresi et al., 2001). Aside from disrupting ionic influx in striatal neurons, 3-NP also enhances glutamate concentration, which results in excitotoxicity (Marti et al., 2003). HD's hyperkinetic symptoms result from an imbalance in neurotransmitter levels in the brain caused by all these reactions together (Binawade and Jagtap, 2013).

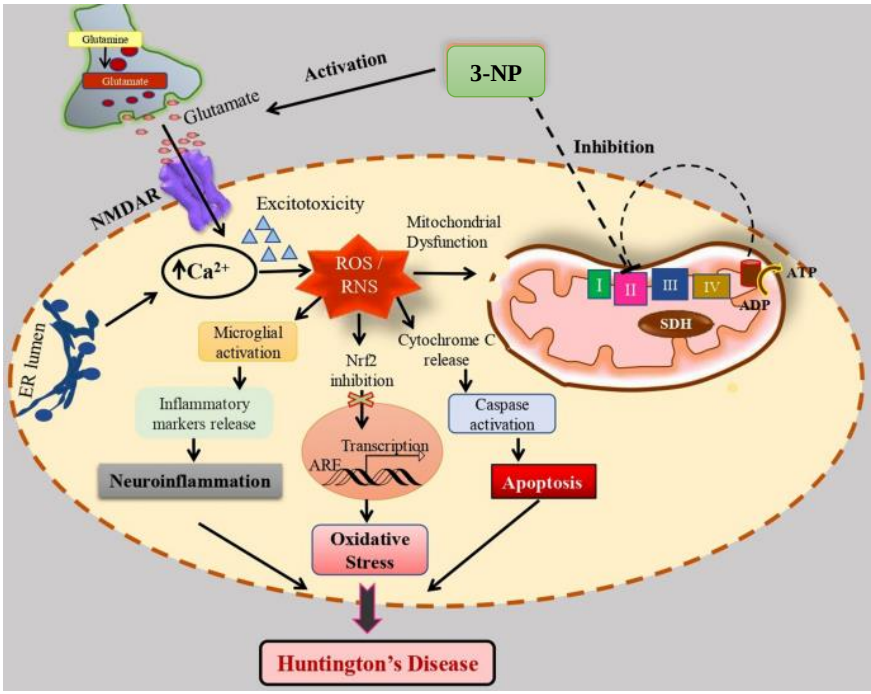

Fig.12. Cellular excitotoxicity and mitochondrial dysfunction caused by 3-NP. The 3-Nitropropionic Acid (3-NP) causes cell death by inhibiting mitochondrial complex II enzymes, disrupting bioenergetics, releasing cytochrome C, which activates caspase, increasing the severity of Huntington's disease (HD). Additionally, 3-NP increases glutamate release, overactivating NMDA receptors and enhancing Ca^{2+} efflux from the ER. As a result, excitotoxicity occurs and reactive oxygen species (ROS) / reactive nitrogen species (RNS) are produced. Inflammation cascades are activated by ROS, which inhibit anti-oxidant production by Nrf2. In addition to raising oxidative stress in the nucleus, this reduces the radical scavenging activity of the cell, leading to striatal nerve degeneration and eventually cell death, contributing to HD. (Image source: Upadhyay et al., 2023)

As a leading cause of neurodegeneration in HD, neuroinflammation plays a key role. The in-vivo study found that 3-NP exposure causes striatal neuroinflammation by causing increased oxidative stress and mitochondrial dysfunction, which, in turn, activates inflammatory cytokines that cause neuroinflammation similar to HD (Jamwal and Kumar, 2016). In addition, 3-NP downregulated Nrf2/ARE signaling, which causes an imbalance of anti-oxidant enzymes that leads to disease progression, as Nrf2/ARE signaling controls the excessive generation of ROS (Gonchar et al., 2021; Silva-Palacios et al., 2017). Researchers have shown that 3-NP impairs MAPK pathway functioning, increases oxidative stress, and accelerates excitotoxicity in rodent brains, resulting in motor abnormalities (Yang et al., 2021). Additionally, it has been shown that 3-NP overactivates

the PI3K/Akt signaling pathway, which leads to oxidative stress, which is associated with striatal neurodegeneration and increases autophagy, which further activates caspases, which decreases BDNF levels in the brain (Kulasekaran and Ganapasam, 2015; Mustafa et al., 2021). These earlier studies suggest that 3-NP exposure also results in a reduction in BDNF protein synthesis, which decreases cell growth, proliferation, synaptic plasticity, and imbalance in cellular homeostasis that is associated with HD (Ranju et al., 2015; Shalaby et al., 2018; Wu et al., 2017). As a result of all these conditions, 3-NP induced models had similar motor abnormalities to HD patients, including involuntary hypokinetic movement, dystonia, and muscle rigidity. As a result, 3-NP HD models could serve as a more effective tool for determining the therapeutic effectiveness of vitamins like VD.

Chapter 2

Protective Role of Vitamin D3 on Motor Dysfunction and Spatial Memory

2.1. Introduction:

Huntington's disease (HD) is a progressive neurodegenerative disorder which is one of the most untreatable pathology in brain which is rising especially in India. The prelavance of the disease is 0.4/1,00,000 population in Asia where as in India, it is which is 1.75/1,00,000 population as per 2020 (Hussain et al., 2020). The disease leads to selective loss of medium spiny neurons (MSNs) which leads to the decrease in motor co-ordination in HD (Lewitus et al., 2014). Loss of MSNs leads to the loss of γ-amino butyric acid (GABA) signaling which leads to involuntary movements, lack of coordination, and cognitive and psychiatric impairments (Gil and Rego, 2008). HD is an autosomal dominant neurodegenerative disorder caused by the expansion of CAG (encodes for glutamine) in Huntingtin gene (*Htt*) located on 4^{th} chromosome with an inverse correlation between the number of repeats and age of onset of disease (Gil-Mohapel, 2012). 3-NP is a mitochondrial toxin leads to striatal damage thereby mimicking the symptoms of HD (Gao et al., 2015). It is an irreversible inhibitor of succinate dehydrogenase of tricarboxylic acid cycle, which has been used to explore the molecular mechanisms related to striatal damage in HD (Túnez et al., 2010).

Calcitriol, the active form of VD is the fundamental agent which helps in maintaining the survival of neurons, synaptic plasticity by its activity on certain agents like brain derived neurotrophic factor (BDNF), acetylcholine, dopamine and GABA (Lustig, 2006; Moretti et al., 2018). It has been evident that, VD deficiency is seen in neurodegenerative diseases like HD (Chel et al., 2013; Fort Molnár et al., 2016). VD is a neurosteroid hormone which shows its protective role in neurodegenerative diseases like Parkinson's and Alzheimer's disease (AlJohri et al., 2019; Banerjee et al., 2015; Calvello et al., 2017; Lima et al., 2018; Mohamed et al., 2015). The neuroprotective role of VD will take place via VDR (Eyles, 2020; Lv et al., 2020; Manjari et al., 2022).

Evidence suggests that, VD supplementation rescues the behavior of animals like locomotion, neuromuscular co-ordination and spatial memory depicting its protective role (Koduah et al., 2017; Lima et al., 2018; Mohamed et al., 2015; Yamini et al., 2018). Though VD supplementation is readily available and affordable, little is known about its potential beneficial effects in HD. Limited evidence is available to correlate VD deficiency with HD

and whether high VD supplementation affects motor function in HD has not been established (Chel et al., 2013). Thus, the present chapter examines the effect of two different doses of VD supplementation on motor dysfunction following the administration of 3-nitropropionic acid (3-NP) and elucidates the optimal dose to determine the protective role.

2.2. Materials and methods:

2.2.1. Animal Procurement:

Ten to twelve weeks old male C57BL/6 mice (average weight; 26 ± 3 g) were acquired from Sainath Agencies, Hyderabad, India. Animals were group housed (2 mice per cage) with *ad libitum* access to food and water. They were kept in a 12 h light/12 h dark cycle at 25±2 °C. All the animal experiments were carried out with the approval of the Institutional Animal Ethics Committee (IAEC), BITS - Pilani, Hyderabad (BITS/Hyd/IAEC/2019/10, BITS/Hyd/IAEC/2020/20). All efforts were made to minimize the number of animals used and their suffering.

2.2.2. Study design:

All the animals were acclimatized for 5 days and then received behavioural training for 7 days prior to treatment. Animals were then randomly divided into 6 experimental groups (Group I to Group VI; Table 5) and given injections of 3-NP and/or VD (cholecalciferol) (**Fig. 13**). 3-NP was given by three intraperitoneal injections of 25 mg/kg, every 12 h, for a cumulative dose of 75 mg/kg as described previously by Amenda et al (2005) and Fernagut et al (2002) with minimal modification (Amende et al., 2005; Fernagut et al., 2002). VD was given i.p. daily for 15 days at two different doses 500IU/kg and 2000IU/kg as shown in the Table 5.

2.2.3. Experimental design:

The mice were randomly divided into four experimental groups for behavior and biochemical assay. (**Table 5**)

i. **Group I:** Control group mice (C57BL/6) injected with saline.

ii. **Group II:** 3-NP induced mice by i.p. injection (3-NP; 75 mg/kg) without VD-treatment (HD).

iii. **Group III:** Mice injected solely with 500IU/kg/day VD for 15 days**.**

iv. **Group IV:** Post-intraperitoneal injection of 500IU/kg/day of VD to 3-NP (75 mg/kg) pre-injected mice for 15 days (HD + VD).

v. **Group V:** Mice injected solely with 2000IU/kg/day VD for 15 days**.**

vi. **Group VI:** Post-intraperitoneal injection of 2000IU/kg/day of VD to 3-NP (75 mg/kg) pre-injected mice for 15 days (HD + VD).

Table 5: The six different experimental groups of C57BL/6 male mice (3-4 months old).

Animal	Group
	**Group I** (Control – 1X saline)
	**Group II** (3-NP-75mg/kg)
	**Group III** (VD-500IU/kg/day for 15 days)
	**Group IV** (3-NP-75mg/kg + VD-500IU/kg/day for 15 days)
	**Group V** (VD-2000IU/kg/day for 15 days)
	**Group VI** (3-NP-75mg/kg + VD-2000IU/kg/day for 15 days)

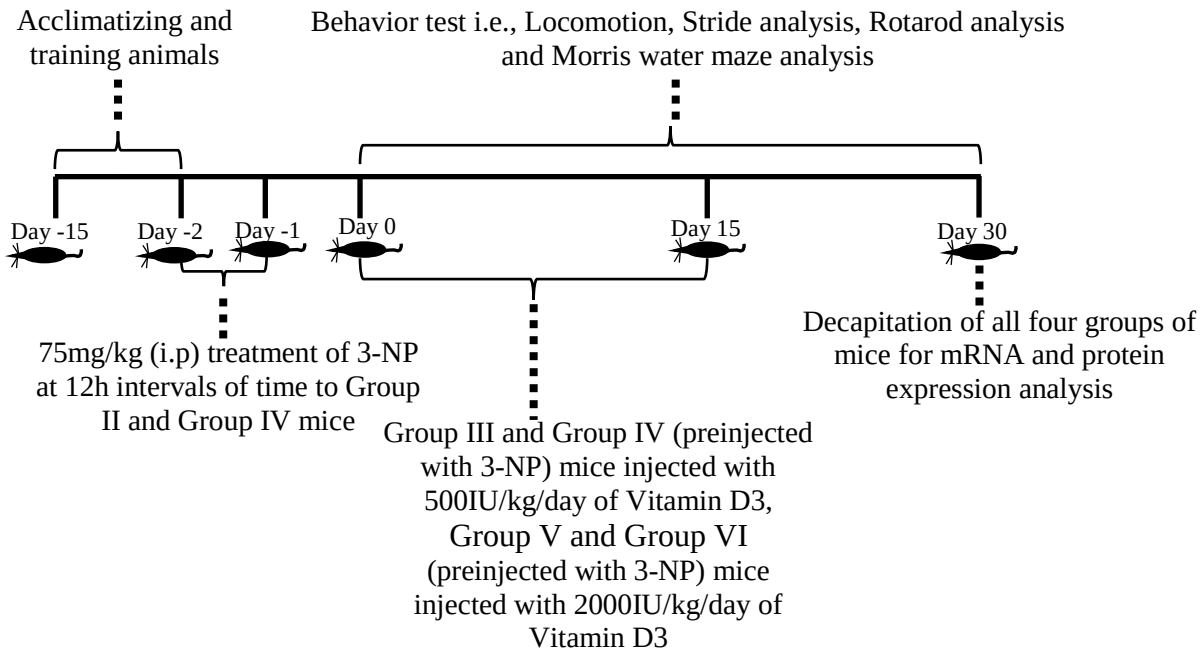

Fig. 13. Timeline of present study

2.2.4. Drugs and reagents:

2.2.4.1 Cholecalciferol:

Cholecalciferol (Vitamin D3; VD) was purchased from Sigma-Aldrich, India (Cat No: C9756) and dissolved in 1% ethanol (diluted with sterile saline) on the day of injection (Mohamed et al., 2015). Mice were administered with two different doses of VD i.e., 500IU/kg/day (12.5µg/kg/day) and 2000IU/kg/day (60µg/kg/day) through intraperitoneal injection reported previously by Kolla and Majagi (Chabas et al., 2013; Gueye et al., 2015; Kolla and Majagi, 2019). Briefly, VD was administered to the Group III and V (only VD) mice and Group IV and VI (HD+VD) mice. Group IV and IV mice (HD + VD) were given 24 hr recovery time from previous 3-NP induction. Then the VD injections were carried out 24 hr after the last dose of 3-NP daily for 15 days to Group IV mice (from 0 to 15th day, **Fig. 13** and **Table 5**).

2.2.4.2. 3-Nitropropionic acid:

3-nitropropionic acid (3-NP) was purchased from Sigma-Aldrich, India (Cat No.: N22908). Stock solutions of 3-NP (3 mg/ml) were prepared in 0.1M phosphate buffered saline solution and were injected intraperitoneally at 25 mg/kg (3-NP; cumulative dose of 75 mg/kg) thrice at 12 h intervals to respective groups of mice as described previously (**Fig. 13** and Table 5). Controls were treated with three doses of saline at 12 h intervals. In this study, we used a subacute dose of 3-NP dose as reported previously by Amenda et al (2005) with minimal modification (Amende et al., 2005). This protocol is based on previous published studies by Fernagut et al. and Kim and Chan who used 50 mg/kg i.p. injection of 3-NP for 5 days. To model a subacute exposure to 3-NP, a cumulative dose of 75 mg/kg dose of 3-NP was undertaken (Fernagut et al., 2002; Kim and Chan, 2001).

2.2.5. Behavioral assessment:

A total of 80 mice were used for behavioral experiments. Mice were initially assessed for locomotion and gait as previously reported (Amenda et al., 2005; Fernagut et al., 2002). A separate cohort was used to evaluate the effects on locomotion and rotorod performance. Only two behavioral tests were done on a given set of animals. Protocols for behavioral tests were:

2.2.5.1. Assessment of locomotor activity:

The locomotor activity was monitored using an actophotometer as described previously (Digital Photoactometer cage; Dolphin, 2009, Kumar et al., 2009), using the number of beam breaks as the measure of movement for each animal. Locomotion was measured over a 5 min period, and baseline readings were taken before the respective drug injections (**Fig. 14**).

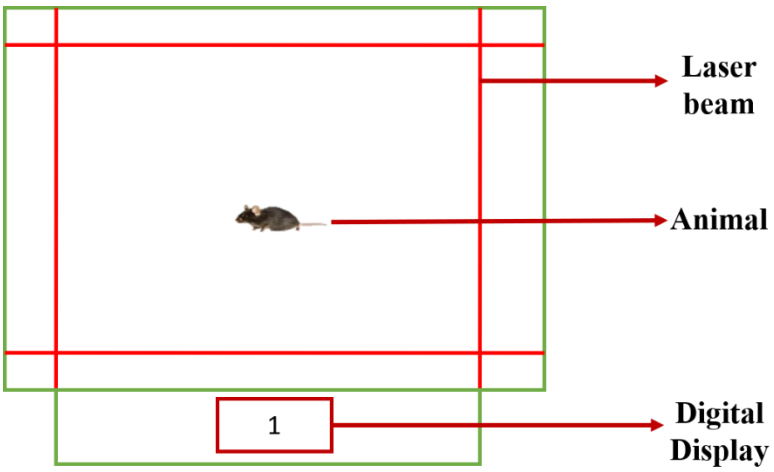

Fig. 14. Schematic representation of locomotor activity by using actophotometer

2.2.5.2. Estimation of gait by stride analysis:

Stride length analysis was done to determine the hyperkinesia in mice by marking the animals' forepaws and hind paws with ink (red for forelimbs and blue for hind limbs; **Fig. 15A and B**). The animals were allowed to move on a strip of paper (4 cm wide and 56 cm long) placed on a brightly lit runway leading to a darkened box. Stride length was measured manually as the distance between two paw prints as described previously (Fernagut et al., 2002). Forelimb stride length measurement was first measured for all mice followed by hind limb stride length on a new strip of paper.

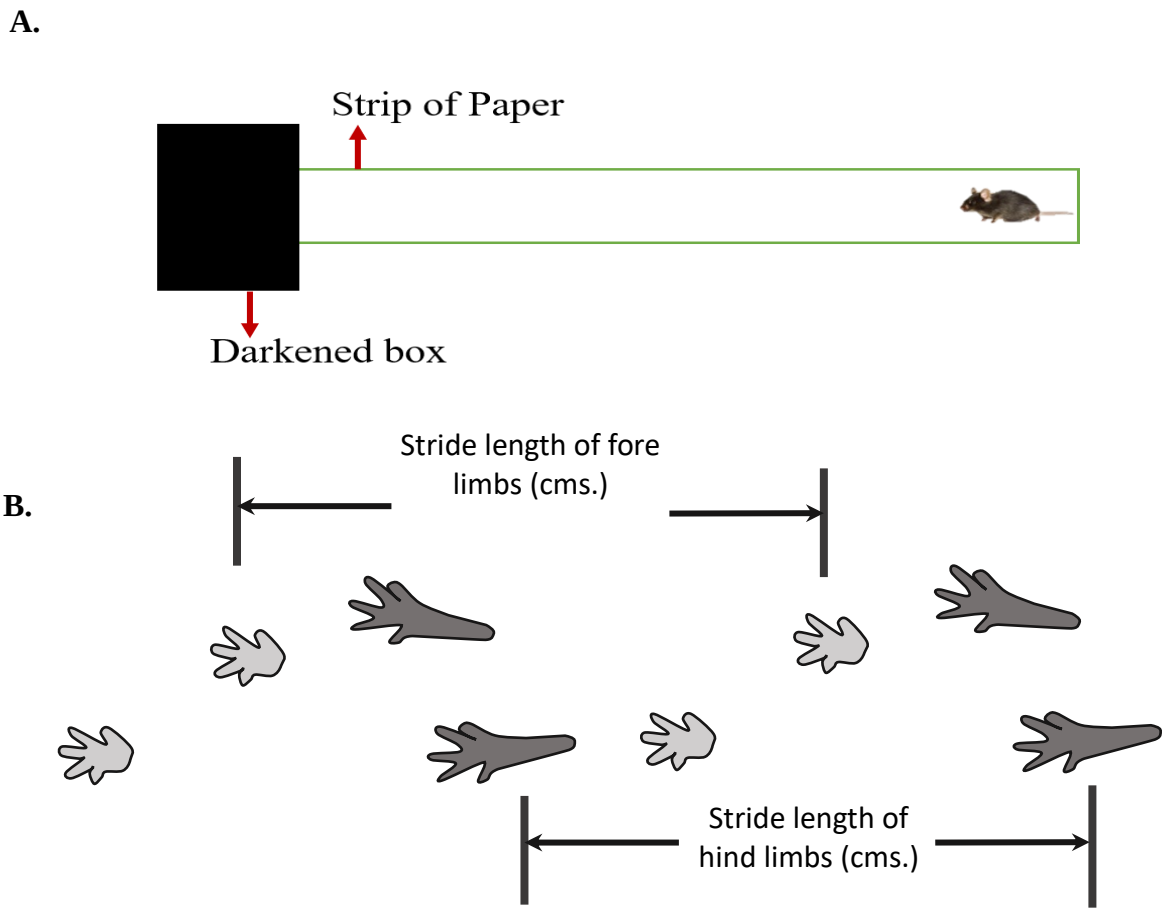

Fig. 15. (A) Schematic representation to carry out the stride length (B) Representation for the estimation of gait which can be analyzed by the average stride of fore limbs and hind limbs.

2.2.5.3. Assessment of motor coordination by rotarod analysis:

The integrity of motor coordination was measured using the rotarod as described previously (Kumar et al., 2009). Briefly, the rotarod apparatus consists of a long rotating rod of 90 cm long and 3 cm in diameter. The apparatus was divided into three different compartments by a glass partition (Rota rod 3 compartments, Dolphin, 2019). The rod rotation speed was set initially at 35 rotations per minute (RPM). Mice received training on the accelerating rod prior to treatment. After achieving criterion (no falls from the rotarod within 180 sec, mice were injected with either saline (Group I; Control), 3-NP (Group II; HD) or VD (Group III) or both (Group IV; HD + VD). After the respective injections, the treated mice were re-tested for 180 sec and the latency to fall was recorded and analyzed.

2.2.5.4. Assessment of Spatial memory by Morris water-maze test:

Morris water-maze (MWM) test was conducted in a white round pool of 92 cm in diameter and 60cm in depth as described previously (Barnhart et al., 2015). The pool was filled to a depth of 40 cm with white opaque non-toxic water-based paint and was divided into 4 quadrants as shown in **Fig. 16**. Pool temperature was maintained at 25±1°C and pH at 7±0.1. An escape platform was placed at the center of the 4th quadrant. The platform was located at 20 cm from the pool's edge and submerged at 1cm beneath the water surface. The platform remained at the same position throughout the training and testing periods. Training over 10 consecutive days was undertaken and consisted of trials during which each animal could escape swimming by finding a permanently located submerged platform within 60 sec (Barnhart et al., 2015). Following training, the animals were divided into four groups. Group 1 animals (control) and group 2 animals (HD mice) received intraperitoneal injections of either 1X saline or 3-NP (75 mg/kg). Group 3 (only VD) and group 4 (3-NP + VD) received intraperitoneal injections of either 500IU/kg of VD or 3-NP (75 mg/kg) together with VD for a dose of 500IU/kg. All animals before being divided into respective groups underwent training. Thereby, the amount of time taken by the animal to locate the platform was recorded manually using a timer and the proportion of escape latency was subsequently measured.

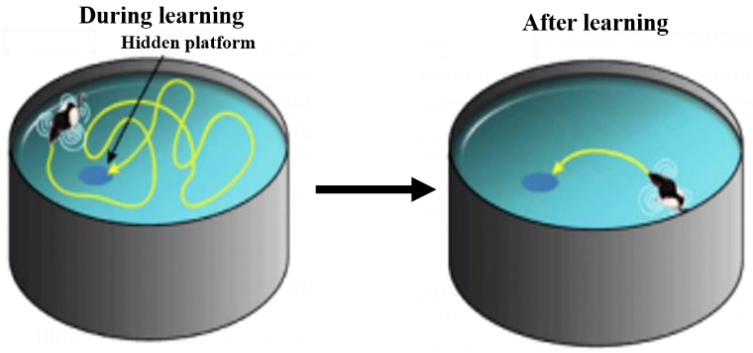

Fig. 16. Diagrammatic representation for the calculation of spatial memory by morris water maze test.

2.2.6. Statistical analysis:

Data is represented as normalized values w.r.t to zero day for the respective groups of mice and reported as mean ± SEM (standard error of the mean). Statistical analysis was conducted using two-way repeated measures ANOVA, two-way ANOVA and one-way repeated measures ANOVA followed by either post hoc multiple pairwise analysis using Tukey's HSD tests or paired sample t-test. For non-parametric measurements, a Kruskal–Wallis test followed by an unpaired sample t-test was performed. $p < 0.05$ was set as threshold of significance ($^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$). All the statistical data was analyzed using Origin 8.1.

2.3. Results:

2.3.1. VD supplementation improves the locomotor activity in the mouse model of HD:

The impact of supplementation of two different doses of VD i.e., 500IU/kg/day and 2000IU/kg/day on the locomotor activity in HD model mice was tested over a period of 30 consecutive days. An actophotometer was used to determine the total number of beam crossings for the evaluation of hypokinesia (**Fig. 14.;** Kumar et al 2009). By the 7th day there were no differences in the movement among the six experimental groups of mice. However, a consistent decrease of roughly 30% in the locomotory activity was observed on the 14th and 21st days in 3-NP injected HD mice (Group II) as compared to Control (Group I) mice; (14th day, 0.76 ± 0.08 vs 1.01 ± 0.11; 21st day 0.66 ± 0.07 vs 1.03 ± 0.05, n = 8-10, $p < 0.001$, Tukey's *post-hoc* analysis, **Fig. 17B**). On the 30th day it further deteriorated to 40% of Control values (Group II vs Group I; 0.40 ± 0.02 vs 1.03 ± 0.06, n = 10, $p < 0.001$, Tukey's *post-hoc* analysis, **Fig. 17B**). However, on the 14th and 21st days, Group IV mice supplemented with 500IU/kg/day of VD and pre-injected with 75 mg/kg of 3-NP showed a rescue in the locomotor activity near control levels and significantly above Group II mice (3-NP treated HD mice) (Group IV vs Group II; 14th day, 0.92 ± 0.09 vs 0.76 ± 0.08; 21st day, 0.83 ± 0.07 vs 0.66 ± 0.07, n = 10, $p < 0.001$, Tukey's *post-hoc* analysis, **Fig. 17A**). But by 14th and 21st day, Group VI mice when supplemented with 2000IU/kg/day did not show any rescue effect when compared with 3-NP treated HD mice (Group VI vs Group II; 14th day, 0.76 ± 0.15 vs 0.76 ± 0.08, n = 12, $p = 0.99$; 21st day, 0.68

± 0.25 vs 0.66 ± 0.07, n = 10, p = 0.93, Tukey's *post-hoc* analysis, **Fig. 17A**). After 21st day surprisingly 2000IU/kg/day of VD when supplemented with HD mice showed rescue effect. On the 30th day, Group IV mice on 500IU/kg/day of VD supplementation showed a robust enhancement by 1.2-fold in the locomotion performance as compared to HD mice (0.86 ± 0.07 vs 0.40 ± 0.02, n = 10, p < 0.001, Tukey's *post-hoc* analysis, **Fig. 17B**). Similarly, by 30th day Group VI mice on 2000IU/kg/day of VD supplementation showed 0.6 fold increase in the locmotor activity when compared with HD mice (0.65 ± 0.19 vs 0.40 ± 0.02, n = 10-12, p = 0.02, Tukey's *post-hoc* analysis, **Fig. 17B**).

The results infer that, by 30th day similar rescue effect was observed by 30th day when supplemented with both the doses of VD for 15 days I.e., 500IU/kg/day and 2000IU/kg/day (Group IV vs Group VI, vs 0.86 ± 0.07 vs 0.65 ± 0.19, n = 10 to 12, p = 0.35, Tukey's *post-hoc* analysis, **Fig. 17B)**. These results confirm that 500IU/kg of VD supplementation as an otimal dose to rescue the locomotion when striatal nsurons were subjected to 3-NP induced neurodegeneration. Furthermore, this data also confirms that the effect of VD supplementation caused no chronic toxic side effect at either of the doses.

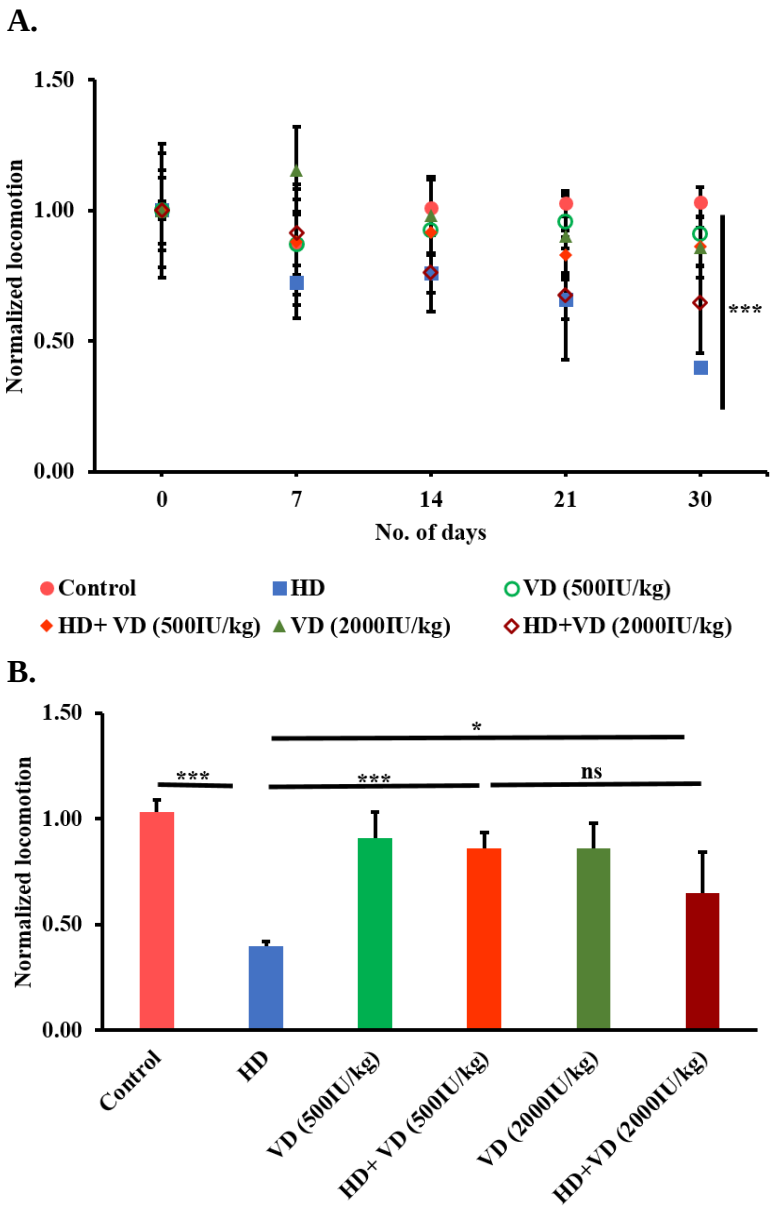

Fig.17. VD supplementation rescues locomotor performance in HD mice. (A) VD supplementation (500IU/kg/day and 2000IU/kg/day) significantly reversed the immobility effect of 3-NP observed in HD mice (n = 8 -12; $p < 0.001$, two-way repeated measures ANOVA). (B) By 30th day, a significant decrease in locomotion activity was observed in HD mice (Group II) as compared to control (Group I), which was reversed significantly by VD induction (Group IV vs Group II, n = 8-10, $p < 0.001$; Group VI vs Group II, n = 10-12, $p = 0.02$; Tukey's *post-hoc* analysis). No significant difference between locomotion activity between the mice supplemented with two different doses of VD (500IU/kg/day and 2000IU/kg/day) to 3-NP induced HD mice (Group IV vs Group VI, n = 8 – 12, $p = 0.35$, Tukey's *post-hoc* analysis). All data are normalized against zero day for respective group and is represented as mean ± SEM.

2.3.2. Gait was unaltered on 3-nitropropionic acid induced mouse model of HD:

To determine the potential neuroprotective role of both doses of VD supplementation (500IU/kg/day and 2000IU/kg/day) on gait of 3-NP treated mice, we measured the distance between two successive paw prints (**Fig. 15**) for four weeks. No change in the stride length was observed across all the six groups of the mice respectively (**Fig. 18**). In comparison with Controls (Group I), HD mice (Group II) gait dynamics remained unchanged for all the respective timepoints (Group II vs Group I; 7^{th} day, 0.90 ± 0.03 vs 0.98 ± 0.04; 14^{th} day, 1.00 ± 0.07 vs 1.02 ± 0.06; 21^{st} day, 0.88 ± 0.11 vs 1.09 ± 0.09; 30^{th} day, 1.06 ± 0.05 vs 1.03 ± 0.05, n = 4 - 10, p = 0.7, Tukey's *post-hoc* analysis, **Fig. 18A**). Even on the 30^{th} day, where we found a highly significant 60% decrease in the locomotion in HD mice (**Fig. 17B**) the gait dynamics remained unaltered between Control and HD mice (Group II vs Group I; 1.06 ± 0.05 vs 1.03 ± 0.05, p = 0.7; n = 10 each, Tukey's *post-hoc* analysis, **Fig. 18B**). Similarly, no effect of either the dose of VD supplementation (500IU/kg/day and 2000IU/kg/day) was seen on stride length in 3-NP treated mice (Group IV and VI) as compared with HD mice (Group II) for entire timeline of the study (Group IV and Group VI vs Group II; 7^{th} day, 0.88 ± 0.09 and 1.08 ± 0.06 vs 0.90 ± 0.03; 14^{th} day, 0.79 ± 0.03 and 1.03 ± 0.06 vs 1.00 ± 0.07; 21^{st} day 0.88 ± 0.02 and 0.92 ± 0.02 vs 0.88 ± 0.11; 30^{th} day, 1.03 ± 0.04 and 1.05 ± 0.06 vs 1.06 ± 0.05, n = 4-10, **Fig. 18A and B**). A one-way balanced repeated measures ANOVA was conducted for the 30^{th} day timepoint to cross check whether VD supplementation modulated gait dynamics in HD mice (**Fig. 18B**). A power analysis done only for the 30^{th} day gave a value of 1. This time point was chosen primarily because we found a robust effect of VD at this time point in other behavior tests (**Fig. 17 and 19**). Consequently, VD supplementation (either alone or in conjunction with 3-NP treatment) also did not impact the stride length performance of the mice across all time points of the present study. Our results agree with the findings of Fernaugut et al (2002) where even a much higher cumulative dose of 3-NP (340 mg/kg) resulted in no differences in stride length for either forelimbs and hind limbs in mice (Fernagut et al., 2002). The data suggest that since the postural gait control is regulated through reciprocal connections between the brainstem and cerebellar cortex, the obtained result may reflect that the dose of 3-NP (75 mg/kg) used in the present study did not possibly produce a significant neuronal loss in the cerebellum (Takakusaki et al 2017).

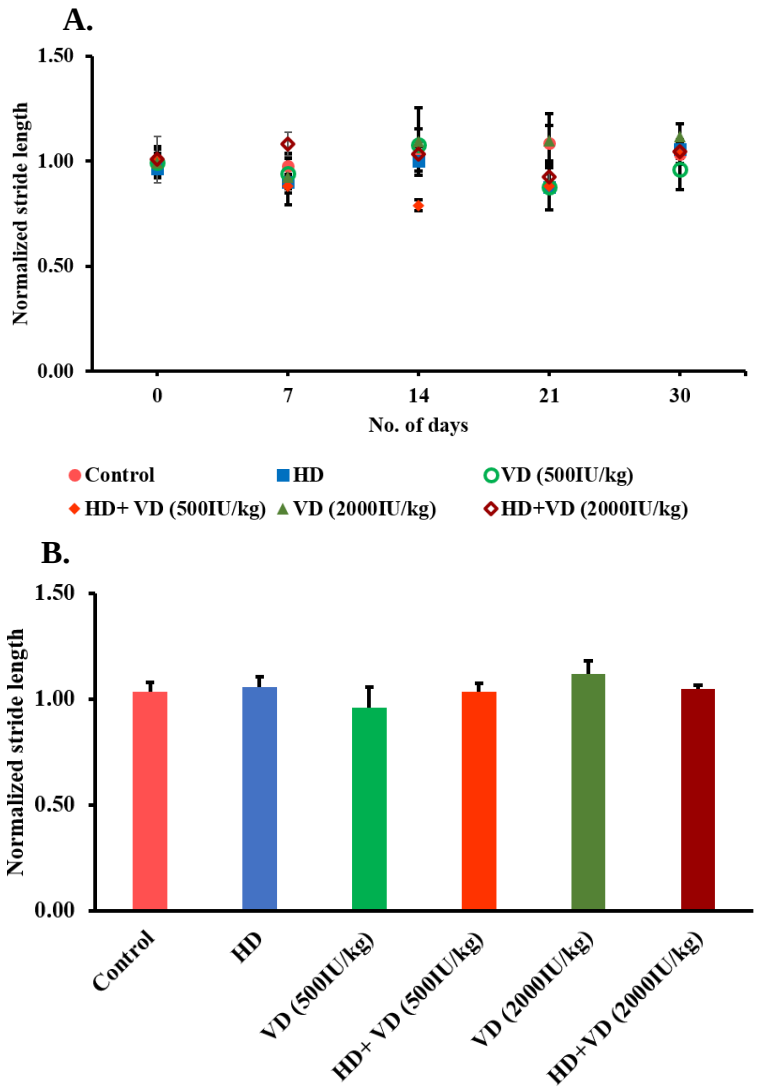

Fig.18. VD administration shows no effect in GAIT dynamics of HD mice. (A) VD supplementation (500IU/kg/day and 2000Iu/kg/day) showed no significant effect on gait dynamics in 3-NP induced HD mice (n = 4 -10, p = 0.4, two-way ANOVA). 3-NP (i.p; 75mg/kg, Group II) induced mice produced no change in fore limb and hind limb performance as compared with Group I (Control) mice across a span of 30 days. (B) On 30th day, no effect of i.p injection of VD (500IU/kg/day and 2000IU/kg/day) was observed in the stride length performance of 3-NP pre-treated mice (Group IV and VI) (n = 10, p = 0.67, Tukey's *post-hoc* analysis). Data is represented as normalized mean ± SEM value against zero day for respective group represent the stride length measurement of each mouse.

2.3.3. VD supplementation improves rotarod performance in HD mice:

To test the potential effect of VD supplementation to rescue grip strength in 3-NP induced HD mice, we used the rotarod to determine the latency of first fall for the evaluation of motor coordination for four weeks (Amende et al 2005, Rodrigues et al 2019). We found that on the 7^{th} day as well as on the 14^{th} day, 3-NP injected HD mice consistently showed around a 50% reduction in fall latency when compared with the aged-matched Control animals (Group II vs Group I; 7^{th} day; 0.63 ± 0.22 vs 1.40 ± 0.15; 14^{th} day, 0.58 ± 0.19 vs 1.33 ± 0.08, n = 8 - 9, $p < 0.001$, two-way ANOVA followed by Tukey's *post-hoc* analysis, **Fig. 19**). On the 21^{st} and 30^{th} days, 3-NP treated mice still had a roughly 35% decrease in the latency to fall as compared to Control mice (Group II vs Group I; 21^{st} day, 0.92 ± 0.22 vs 1.40 ± 0.05; 30^{th} day, 0.90 ± 0.22 vs 1.45 ± 0.001, n = 8 - 9, $p < 0.001$, two-way ANOVA followed by Tukey's *post-hoc* analysis, **Fig. 19A and B**). A significant improvement in the neuromuscular coordination was observed between Group IV mice (HD + VD) and Group II mice (HD) from the 7^{th} day onwards and continued through the 30^{th} day (**Fig 19A and B**). Astonishingly, Group IV (HD + VD) mice showed a highly significant effect of VD supplementation on rotarod performance on the 14^{th} day by 1.4 fold (1.37 ± 0.13), on the 21^{st} day by 0.6-fold (1.44 ± 0.1) and on the 30^{th} day by 0.74 fold (1.57 ± 0.001) as compared to Group II mice (HD) for the same time points (14^{th} day, 0.58 ± 0.19; 21^{st} day, 0.92 ± 0.22; 30^{th} day, 0.90 ± 0.22, n = 8 - 9, $p < 0.001$, Tukey's *post-hoc* analysis, **Fig. 19A and B**). To our surprise VD treatment to pre-3-NP injected mice (Group IV; HD + VD) recorded no latency to fall within a total time duration of 180 seconds and rescued the neuromuscular coordination by 100% when compared with 3-NP induced HD mice. To rule out the possibility that VD supplementation alone showed any effect on the grip strength of mice, VD injections were carried out in a Control group (Group III). Interestingly, we found no significant difference in the latency to first fall between the Group I (Control) and the VD supplemented mice (Group III) for all time points ($p = 0.9$; Tukey's *post-hoc* analysis, **Fig. 19A and B**). Overall, two-way ANOVA showed a significant difference in the mean among all groups of mice with no interaction between the groups and day ($p < 0.001$, two-way ANOVA, **Fig. 19A**), reflecting the effects of VD and 3-NP in Group II and Group IV mice. These result support our hypothesis that the VD supplementation has a robust rescue effect on neuromuscular coordination, which is

sustained throughout the timeline of the study. Neuromuscular coordination is impaired in patients with HD but how VD might affect the HD associated behavioral performance is not well described in the mouse model (Chel et al., 2013). Our data parallels the findings of Sakai and colleagues who showed that an oral supplementation of the VD analogue eldecalcitol (ED-71, ELD), a derivative of 1,25 $(OH)_2D_3$, for 14 days significantly improved the locomotor performance of mice (Sakai et al., 2015). Here we used a similar dose of VD (500IU/kg/day; 12.5µg/kg/day) for a similar about of time (here 15 days) to explore the motor benefits of VD (cholecalciferol) in HD mice. Our findings collectively suggest that motor performance deficits observed in the 3-NP mouse model of HD get significantly reversed by VD supplementation, suggesting a neuroprotective function of VD in the striatum.

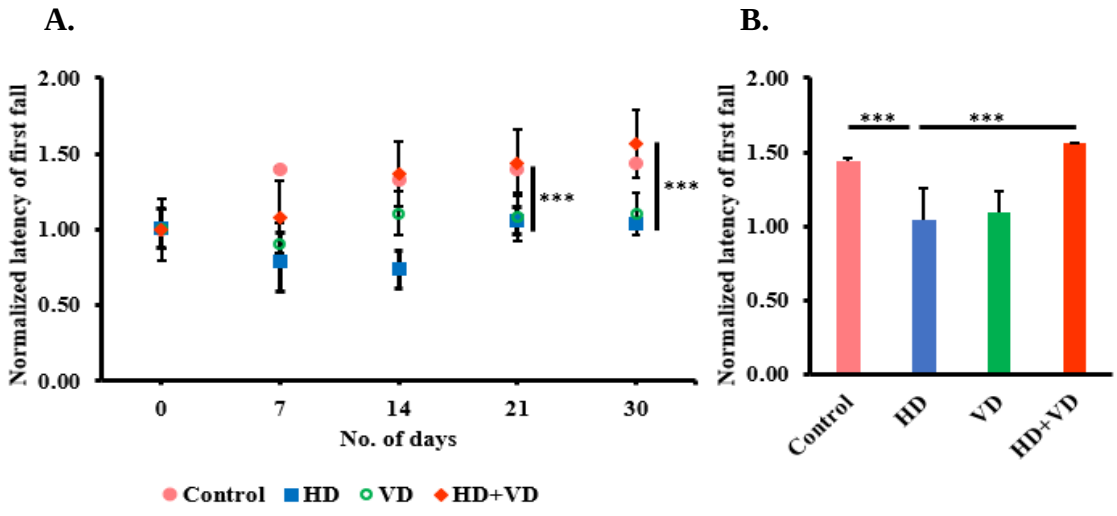

Fig.19. Rotarod performance of HD mice depicting beneficial effect of VD administration in HD mice. (A) Grip-strength of 3-NP induced HD mice was significantly improved on VD supplementation ($p < 0.001$, n = 8-9, two-way ANOVA. (B) On 30th day, HD mice post supplemented with 500IU/kg of VD (Group IV) showed no latency in fall for entire 180 sec from the rotating rod, as compared HD mice (Group II, n = 8, $p < 0.001$, Tukey's *post-hoc* analysis). HD mice induced with 3-NP showed a significant decrease in the as compared to Control (Group I) (n = 8-9, $p < 0.001$, Tukey's *post-hoc* analysis). Data is represented as normalized mean ± SEM value against zero day for respective group and circle represent the latency of the first fall.

2.3.4. VD rescues spatial memory of HD mice:

To test the potential effect of VD supplementation (500IU/kg) on spatial memory function of 3-NP induced HD mice, we used the Morris water maze (MWM) to determine the escape latency of the animal for month duration. Training over a period of 10 consecutive days was given to the animals during which animals had to escape towards the submerged platform within 60 secs before inducing the animals with HD. The time taken by the 4 groups of animals was recorded to locate the hidden for the evaluation of rescue in spatial memory (**Fig. 16**). In agreement with our above behavior data, we noticed that on the 30^{th} day, 3-NP induced HD animals (75mg/kg, group 2) showed nearly a 3-fold increase in the time to locate the hidden platform (0.68 ± 0.18, $n = 9$) when compared with the aged-matched control animals (0.11 ± 0.01, $n = 10$, $p < 0.001$, Tukey's *post-hoc* analysis, **Fig. 20**). On the other hand, VD treatment on post 3-NP injection given to group 4 animals (3-NP + VD) showed significant decrease in the normalized escape latency of animals by 1.8-fold (0.19 ± 0.03, $n = 7$) when compared with 3-NP induced HD animals ($p < 0.001$, Tukey's *post-hoc* analysis, **Fig. 20**), and were indistinguishable from control animals. A similar rescue effect of VD on spatial memory was also observed on 21^{st} day. We observed no significant difference in the escape latency between the control and only VD supplemented animals for the entire time course (**Fig. 20**). Spatial learning and MWM performance depend upon the coordinated action of different brain regions constituting a functionally integrated neural network, and thus our data likely reflects that VD supplementation possibly rescues the co-ordination of striatum with various brain region including cortex, hippocampus and cerebellum (D'Hooge and De Deyn, 2001). Furthermore, this data also confirms that intake of VD alone shows no improvement in cognitive performance as the animals supplemented with only VD (group 3) show no difference in MWM performance when compared to that of aged matched control animals (group 1; **Fig. 20**).

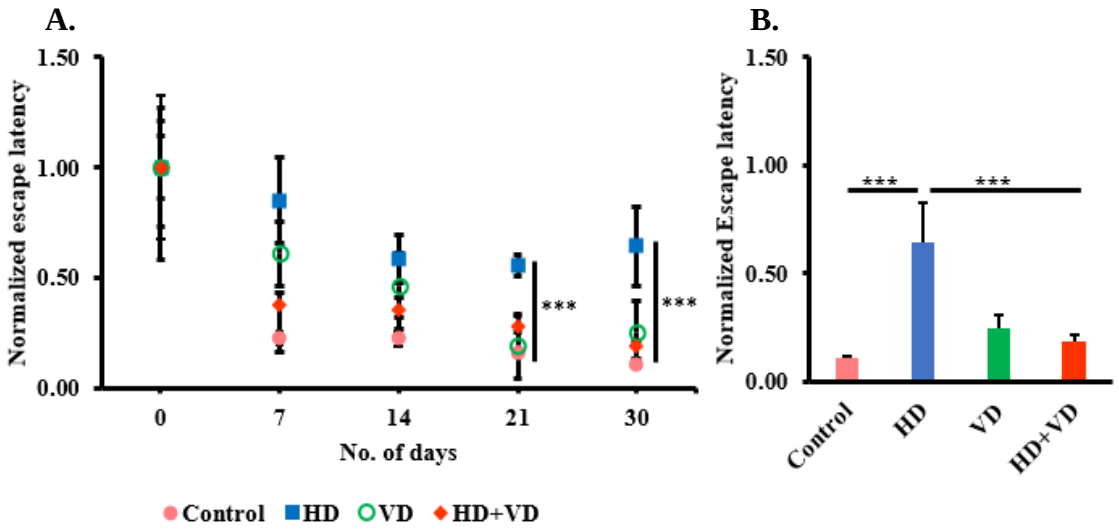

Fig.20. Morris water maze analysis of HD mice depicting beneficial effect of VD administration in HD mice on spatial memory. (A) Memory of 3-NP induced HD mice was significantly improved on VD supplementation ($p < 0.001$, n = 7-10, two-way ANOVA. (B) On 30th day, HD mice post supplemented with 500IU/kg of VD (Group IV) showed minimal escape latency toward the hidden platform, as compared HD mice (Group II, n = 9, $p < 0.001$, Tukey's *post-hoc* analysis). HD mice induced with 3-NP showed a significant increase in escape latency when compared to Control (Group I) (n = 8-9, $p < 0.001$, Tukey's *post-hoc* analysis). Data is represented as normalized mean ± SEM value against zero day for respective group and circle represent the escape latency towards the hidden platform.

2.3.5. VD supplementation maintains the body weight of HD mice:

An overall significant difference in mean body weight was observed among all the groups of mice ($p = 0.002$, two-way ANOVA, **Fig. 21A**). A 30% decrease in the body weight was observed by 30th day in Group II mice when compared with Group I mice (HD vs Control; 0.87 ± 0.01 vs 1.23 ± 0.05, n = 8-10, $p < 0.001$, paired sample t-test, **Fig. 21B**). The body weight was significantly rescued on VD supplementation in HD mice (HD + VD vs HD; 1.10 ± 0.05 vs 0.87 ± 0.01, n = 8-10, $p < 0.001$, paired sample t-test, **Fig. 21B**), possibly reflecting the effect of VD in fixing oxidative stress, mitochondrial function, and muscle heath (Chabas et al., 2013; Kim and Chan, 2001; Latham et al., 2021).

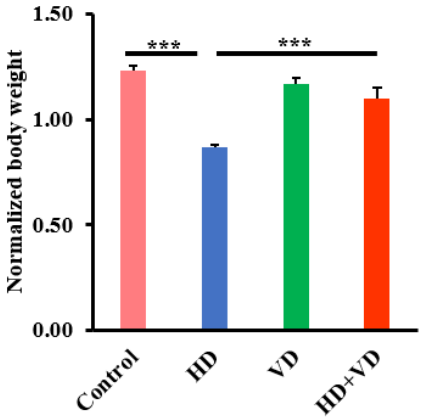

Fig.21. VD administration recues body weight in HD mice. On 30th day, 500IU/kg/day of VD injection to pretreated 3-NP mice (HD) showed a significant rescue in the body weight (Group IV vs Group II, n = 8-10, p = 0.001, paired sample t-test). HD mice showed a dramatic decrease in the body weight as compared to Control (Group II vs Group I, n = 8-10, p < 0.001, paired sample t-test). Data is represented as normalized mean ± SEM value against control.

2.4. Discussion:

Striatum is the main information processing hub of basal ganglia and performs multiple functions including control of movement, reward, and addiction. Dysfunction and death of striatal neurons are the main causes for the motor disorders associated with HD (Lewitus et al., 2014). Though some of the results remain inconclusive, the limited information available suggests a neuroprotective function of VD in the context of the motor dysfunction observed in HD. The goal of the present study was to explore the therapeutic potential of VD in an animal model of HD induced by intraperitoneal injection of 3-nitropropionic acid (3-NP). 3-NP is a well-established toxic model causing mitochondrial dysfunction and selective loss of striatal neurons (Brouillet, 2014; Túnez et al., 2010). In this study, we used a subacute dose of 3-NP, a slight modification from the previous study (Amenda et al., 2005). The protocol is derived from earlier studies by Fernagut and team and the work by Kim and Chan where 50 mg/kg of 3-NP was given for 5 days (Amende et al., 2005; Fernagut et al., 2002, Kim and Chan, 2001). As described by Nishino and team, a single low dose injection of 3-NP (20 mg/kg) was insufficient to induce behavioral and biochemical abnormalities in the striatum but subsequent injections caused significant striatal lesions and motor deficits (Nishino et al., 1997). Our data show that 500IU/kg/day

and 2000IU/kg/day dose (taking an average weight of animals ~ 30gms across the group) of VD given to 3-NP mice produces significant improvement in motor and memory test performances like locomotion, rotarod and Morris water maze as in comparison with 3-NP induced group of animals.

These studies suggested that 500IU/kg/day (12.5µg/kg) of VD improved myelination and accelerated functional recovery of nerve post injury (Chabas et al 2013). In another study, 500IU/kg/day of VD significantly improved the locomotion performance of rodents in a spinal cord injury model that was not observed with a dose of 200IU/kg/day (Gueye et al., 2015). Further, Rodrigues and collegues demonstrated that in rodent model of sporadic dementia of Alzheimer's type, 500IU/kg/day of VD was enough to reduce oxidative stress markers and restore cholinergic function by decreasing acetylcholine esterase activity in synaptosomes (Rodrigues et al., 2019). Based on these findings, we utilized the chronic administration of 500IU/kg/day for 15 days in order to explore its effect on motor disabilities in the 3-NP induced mouse model of HD. We also tested if any benefits were maintained over the next 15 days in the absence of continued VD administration, and our data supported that this is the case.

The rescue effect of VD administration in 3-NP induced HD mice were tested on movement impairment, stride length and grip strength to evaluate the motor coordination of the animals (Beal et al., 1993). Group II mice (3-NP induced) showed a reduction in their latency of fall on the rotarod, whereas Group IV mice (HD + VD) rescued neuromuscular coordination and showed no latency of first fall within a total time duration of 180 seconds as shown in **Fig. 19**. Neuromuscular coordination is known to be impaired in patients with HD but how VD affects this behavior performance in HD have not been described in mouse model (Chel et al., 2013). The findings of the present study suggest that the motor performance deficits observed in the 3-NP model of HD were significantly reversed by VD supplementation, suggesting a neuroprotective function of VD in the striatum. We observed no variability in the gait dynamics across all the four groups (Group I-IV) over a month's time as shown in **Fig. 19 and 20**, possibly reflecting that the dose of 3-NP (75 mg/kg) used in the present study did not produce neuronal loss in the cerebellum (Takakusaki, 2017). Hence, no rescue effect of VD was observed in 3-NP injected HD

mice (**Fig. 19 and 20**). Changes in gait or postural control could occur with different doses or schedules of neurotoxin (3-NP) injection than those undertaken in the present study.

The enhancement in locomotory and rotarod performances of HD mice post injected with VD (Group IV; HD + VD) (**Fig. 17 and 19**). 3-NP induction also significantly decreased the body weight of HD mice (Group II) by ~ 0.3-fold as previously reported by Kumar and team, which was reversed upon VD supplementation by the end of 30 days (HD + VD; Group IV, **Fig. 21**) (Kumar et al., 2009).

The findings in the present chapter reveal that administration of VD following induction of the 3-NP model of HD rescued the impaired motor coordination and locomotion. Overall, VD supplementation has proved to be effective in reversing motor deficits and spatial memory in the 3-NP induced mouse model of HD. It could be considered as promising agents for the development of new therapeutics for neurodegenerative disorders including HD.

Chapter 3

Restorative action of Vitamin D3 on enhancing neurotrophins and antioxidant expression in the striatum via Vitamin D receptor

3.1. Introduction:

From the previous chapter it is evident that 500IU/kg as the optimal dose to study but the molecular mechanism for the rescue in the behavior of mice is still unknown. In the last decade, a potential link has been explored between VD deficiency and neurodegenerative disorders (Amrein et al., 2020; Chel et al., 2013; Holick et al., 2011; Koduah et al., 2017). VD is a neurosteroid hormone that shows neuroprotection effects in animal and cell-culture models of Parkinson's and Alzheimer's disease (Bivona et al., 2019; Calvello et al., 2017; Kim et al., 2006; Nimitphong et al., 2021; Rodrigues et al., 2019). Calcitriol, which is the active form of VD, exerts its neuroprotective role via VDR (Taniura et al., 2006).

Various evidences on other neurodegenerative diseases showed that, VD supplementation has showed upregulation of nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) which helps in the survival of existing neurons (Gao et al., 2022; Mohamed et al., 2015; Wang et al., 2023). These neurotrophins also promote synaptic function and survival of several neuronal populations, including striatal neurons that are the primary affected cells in HD (Zuccato et al., 2001; Zuccato and Cattaneo, 2007). Oxidative stress is also considered as one of the key players for the disease progression in HD (Paul and Snyder, 2019; Túnez et al., 2010). However, studies suggest that, VD has potential role in regulation of oxidative stress during neuropathological conditions which leads to the survival of existing neurons (Lima et al., 2018; Molinari et al., 2019; Wang et al., 2023). This can be identified by the effect of oxidative stress on certain antioxidants like superoxide dismutase (SOD), glutathione peroxidase (GpX), and catalase (Cat). Studies suggest that VD supplementation has a regulatory effect on oxidative stress which leads to the survival of neurons (Bakhtiari-Dovvombaygi et al., 2021; Lima et al., 2018). There is a limited evidence shows that VD supplementation shows its protective role in HD however the molecular mechanism of its therapeutic role is not established (Fort Molnár et al., 2016). Therefore, the present study was focused on the protective role of 500IU/kg of VD supplementation on HD in 3-NP induced mouse. Therefore, the present chapter focusses on the effect of 500IU/kg of VD supplementation

on neuronal survival by its activity on neurotrophins and anti-oxidants through non-genomic action in HD on the administration of 3-nitropropionic acid (3-NP).

3.2. Materials and methods:

3.2.1. Animal Procurement:

Ten to twelve weeks old male C57BL/6 mice (average weight; 26 ± 3 g) were acquired from Sainath Agencies, Hyderabad, India. Animals were group housed (2 mice per cage) with *ad libitum* access to food and water. They were kept in a 12 h light/12 h dark cycle at 25±2 °C. All the animal experiments were carried out with the approval of the Institutional Animal Ethics Committee (IAEC), BITS - Pilani, Hyderabad (BITS/Hyd/IAEC/2019/10, BITS/Hyd/IAEC/2020/20). All efforts were made to minimize the number of animals used and their suffering.

3.2.2. Study design:

All the animals were acclimatized for 5 days and then randomly divided into 4 experimental groups (Group I to Group IV; Table 5) and given injections of 3-NP and/or VD (**Fig. 13**). 3-NP was given by three intraperitoneal injections of 25 mg/kg, every 12 h, for a cumulative dose of 75 mg/kg as described previously by Amenda et al (2005) and Fernagut et al (2002) with minimal modification (Amende et al., 2005; Fernagut et al., 2002). 500IU/kg/day of VD was given i.p. daily for 15 days as shown in the chapter 2.

3.2.3. Experimental design:

The mice were randomly divided into four experimental groups for behavior and biochemical assay as in Chapter 2

i. **Group I:** Control group mice (C57BL/6) injected with saline.
ii. **Group II:** 3-NP induced mice by i.p. injection (3-NP; 75 mg/kg) without VD-treatment (HD).
iii. **Group III:** Mice injected solely with 500IU/kg/day VD for 15 days**.**
iv. **Group IV:** Post-intraperitoneal injection of 500IU/kg/day of VD to 3-NP (75 mg/kg) pre-injected mice for 15 days (HD + VD).

3.2.4. Drugs and reagents:

3.2.4.1 Cholecalciferol:

Cholecalciferol (Vitamin D3; VD) was purchased from Sigma-Aldrich, India (Cat No: C9756) and dissolved in 1% ethanol (diluted with sterile saline) on the day of injection (Mohamed et al., 2015). Mice were administered with two different doses of VD i.e., 500IU/kg/day (12.5µg/kg/day) through intraperitoneal injection reported previously by Kolla and Majagi (Chabas et al., 2013; Gueye et al., 2015; Kolla and Majagi, 2019). Briefly, VD was administered to the Group III and V (only VD) mice and Group IV and VI (HD+VD) mice. Group IV and IV mice (HD + VD) were given 24 hr recovery time from previous 3-NP induction. Then the VD injections were carried out 24 hr after the last dose of 3-NP daily for 15 days to Group IV mice (from 0 to 15th day, **Fig. 13** and **Table 5**).

3.2.4.2. 3-Nitropropionic acid:

3-nitropropionic acid (3-NP) was purchased from Sigma-Aldrich, India (Cat No.: N22908). Stock solutions of 3-NP (3 mg/ml) were prepared in 0.1M phosphate buffered saline solution and were injected intraperitoneally at 25 mg/kg (3-NP; cumulative dose of 75 mg/kg) thrice at 12 h intervals to respective groups of mice as described previously (**Fig. 13** and **Table 5**). Controls were treated with three doses of saline at 12 h intervals. In this study, we used a subacute dose of 3-NP dose as reported previously by Amenda et al (2005) with minimal modification (Amende et al., 2005). This protocol is based on previous published studies by Fernagut et al (2002) and Kim and Chan (2001) who used 50 mg/kg i.p. injection of 3-NP for 5 days. To model a subacute exposure to 3-NP, a cumulative dose of 75 mg/kg dose of 3-NP was undertaken (Fernagut et al., 2002; Kim and Chan, 2001).

3.2.5. RNA isolation and cDNA synthesis:

On the 30th day, mice from respective groups were anesthetized using isoflurane (Rx, NoB506) and immediately decapitated for the extraction of striatal brain samples. Brain tissue was placed into 1 ml of RNAiso PLUS (Takara Bio) and sonicated on ice. 200µl of chloroform was added and samples were centrifuged for 30 minutes at 12,000g at 4°C (Eppendorf Refrigerated centrifuge, 542R). After isolation of the aqueous

phase, an equal volume of isopropanol (Hi-Media Laboratories, Molecular biology grade, India) was added, incubated overnight at -20°C and again centrifuged at 12,000g for 30 minutes at 4°C. Samples were washed with 70% ice-cold ethanol and the obtained pellet was resuspended in nuclease-free water. DNase I (EN052, Thermo Scientific^{TM}, USA) treatment was performed to remove any DNA contamination. DNase-treated samples were made up to 400µl using nuclease-free water. It was followed by sample purification using 1/10th volume of 3M sodium acetate and 2X volume of phenol: chloroform: isoamyl alcohol (Sisco Research Laboratories Pvt. Ltd., India) and centrifuged for 2 minutes at maximum speed at 4°C. The aqueous phase was isolated with addition of an equal volume of ice-cold 100% ethanol, followed by overnight incubation at -20°C. The samples were again centrifuged at maximum speed for 15 minutes at 4°C, then washed with 70% ice-cold ethanol and the obtained pellet was resuspended in nuclease-free water. The total concentration of purified RNA was estimated by the Nanodrop spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA). An equal amount of RNA from each group was used to reverse transcribe complementary DNA (cDNA) with the help of the Verso cDNA synthesis kit (Cat No: AB1453A, Thermo ScientificTM, USA) as per manufacturer's instruction. Briefly, 500 ng of purified RNA was taken from each group for cDNA synthesis with the following reaction conditions: 42°C for 1 h followed by 95°C for 2 minutes. The obtained cDNA was used for semiquantitative PCR.

3.2.6. Analysis of mRNA expression of nerve growth factor, and antioxidant markers by semi-quantitative PCR:

The sequences of neurotrophic genes (nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF)) of the mouse genome were obtained from NCBI. The sequences were deposited in the IDT primer quest tool to get the most suitable primer for gene analysis. For antioxidant marker genes, we analyzed superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), catalase (Cat) and glutathione peroxidase 4 (GpX4). All the genes, primer sequences and amplicon sizes are listed in **Table 6**.

Table 6. Sequence of Primers used in semi-quantitative and real time PCR studies

Gene	Orientation	Sequence of primers (5' to 3')	Amplicon size
18s	Forward	ACGGAAGGGCACCACCAGGA	127
	Reverse	CACCACCACCCACGGAATCG	
NGF	Forward	GGCAGAACCGTACACAGATAG	88
	Reverse	TGTGTCAAGGGAATGCTGAA	
BDNF	Forward	TCCTAGAGAAAGTCCCGGTATC	94
	Reverse	GCAGCCTTCCTTGGTGTAA	
SOD1	Forward	CAGAAGGCAAGCGGTGAAC	107
	Reverse	CAGCCTTGTGTATTGTCCCCATA	
SOD2	Forward	TCCTAGAGAAAGTCCCGGTATC	112
	Reverse	GCAGCCTTCCTTGGTGTAA	
GPx4	Forward	GCCCAATACCACAACAGTAGA	108
	Reverse	CCTGAACCACAGCGATGAA	
Cat	Forward	AATTGCCTCCACACCTTCAC	107
	Reverse	TCACCAAGCTGCTCATCAAC	

Semiquantitative-PCR was performed using respective cDNA with gene specific primers to estimate the relative quantification of target genes. We used the following PCR condition to amplify NGF using 2X PCR master mix (Takara Bio) and 0.5μM of each primer: 95°C for 2 min; 35 cycles of 95°C for 30 sec., 62°C for 30 sec., 72°C for 30 sec.; and a final step of extension of 72°C for 5 min. For antioxidant markers the PCR condition: 95°C for 5 min; 35 cycles of 95°C for 30 sec, 60°C for 45 sec., 72°C for 45 sec.; and a final step of extension at 72°C for 10 min for SOD1, SOD2, and GpX4 whereas Cat amplification was carried out at 56°C for 45 sec. The PCR products were checked by electrophoresis on 1.5% agarose gel, visualized and quantified using Image software by keeping 18s rRNA as a Control (housekeeping gene).

$$Relative\ Quantification\ of\ a\ gene = \frac{Quantity\ of\ the\ required\ gene}{Quantity\ of\ Housekeeping\ gene}$$

3.2.7. Quantitative analysis of brain derived neurotrophic factor by Real PCR:

The expression of brain derived neurotrophic factor (BDNF) among the four groups of mice was assessed by Real time-PCR (RT-PCR) in a CFX96 Touch Real-time PCR system (BioRad) using the GoTaq qPCR SYBR master mix (Cat No #A6001, Promega Corporation). The reaction mixture was prepared according to the manufacturer's protocol using ~12 ng of the cDNA template. Relative gene expression was quantified using the ΔCT method with respective primers (*BDNF* forward 5′-TCCTAGAGAAAGTCCCGGTATC-3′; reverse 5'-GCAGCCTTCCTTGGTGTAA-3') and normalized to *18s* (forward 5'-ACGGAAGGGCACCACCAGGA-3'; reverse 5'-CACCACCACCCACGGAATCG-3'). The gene expression analysis was carried out by ΔΔCT method to determine the fold changes in the expression of BDNF as follows:

$$Fold\ change = 2\text{^-}\Delta\Delta Ct$$

$$\Delta\Delta Ct = \Delta Ct\ (Gene\ of\ interest) - \Delta Ct\ (Housekeeping\ gene)$$

$$\Delta Ct\ (cycle\ difference) = Ct\ (target\ gene) - Ct\ (Control\ gene)$$

3.2.8. Protein expression of VDR by Western blot:

On the 30th day, striatal brain tissue was extracted from all four groups of mice. The tissue was homogenized in the lysis buffer (150 mM sodium chloride, 1.0% TritonX-100, 0.5% sodium dodecyl sulfate and 50mM Tris, pH 8.0). The protein concentration was determined using a Bradford protein assay kit (Bio-Rad, USA). We loaded equal amounts of protein (25 μg) run in a 12% gel, and then transferred to PVDF (Pall Corporation) membrane through a trans blot wet transfer system (Bio-Rad). The membrane was blocked using 5% BSA and incubated with respective primary and secondary antibodies for β-Actin Rabbit mAb (1:3000, CST#4970, Cell Signaling Technology); VDR Rabbit mAb (1:1500, CST#12550, Cell Signaling Technology); Anti-rabbit IgG-HRP-linked antibody (1:5000, CST#7074, Cell Signaling Technology). β-Actin served as a loading control. The signal intensities of the bands were captured using the fusion pulse gel documentation system

(Eppendorf, USA). ImageJ software was used to quantify the band intensities and the protein expression of the protein was determined by using the following formula:

$$Relative\ expression\ of\ protein = \frac{Relative\ band\ intensity\ of\ respective\ group}{Relative\ band\ intensity\ of\ Control}$$

3.2.9. Statistical analysis:

Experimental data are represented as normalized values w.r.t to control. Data is represented in bar plot by illustrating the distribution of normalized values for each respective group of mice (Group I to Group IV). Group data in the text are presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted using one-way ANOVA followed by either post hoc multiple pairwise analysis using Tukey's HSD tests or paired sample t-test. $p < 0.05$ was set as threshold of significance ($^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$). Statistical analysis was performed using Origin 8.1.

3.3. Results:

3.3.1. VD supplementation increases the expression of neurotrophins in 3-nitropropionic induced Huntington's mice:

3.3.1.1. VD supplementation enhances the gene expression of nerve growth factor in HD mice:

To test the possibility whether VD supplementation upregulated survival pathways in the striatal neurons following 3-NP injection, we measured mRNA expression (see methods) of nerve-growth factor (NGF) from the striatum. On the 30th day we found 3-NP injected HD animals showed a profound reduction by ~36% in the expression of NGF from striatum (group 2, 0.66 ± 0.04, n = 4, **Fig. 22A**) when compared to control mice (group 1, 1.00 ± 0.00, n = 4, $p < 0.0001$, student's t-test, **Fig. 22A**). NGF expression was increased by 72% (group 4, 1.14 ± 0.12, n = 4) when compared with 3-NP induced HD animals (group 2, 0.66 ± 0.04, n = 4, $p = 0.004$, student's t-test, **Fig. 22A**) in the striatum. Furthermore, mRNA expression of NGF remain unchanged between mice supplemented with only VD and control. These results confirmed that VD supplementation can enhance

the expression of NGF only when striatal neurons are subjected to neurodegeneration on 3-NP induction.

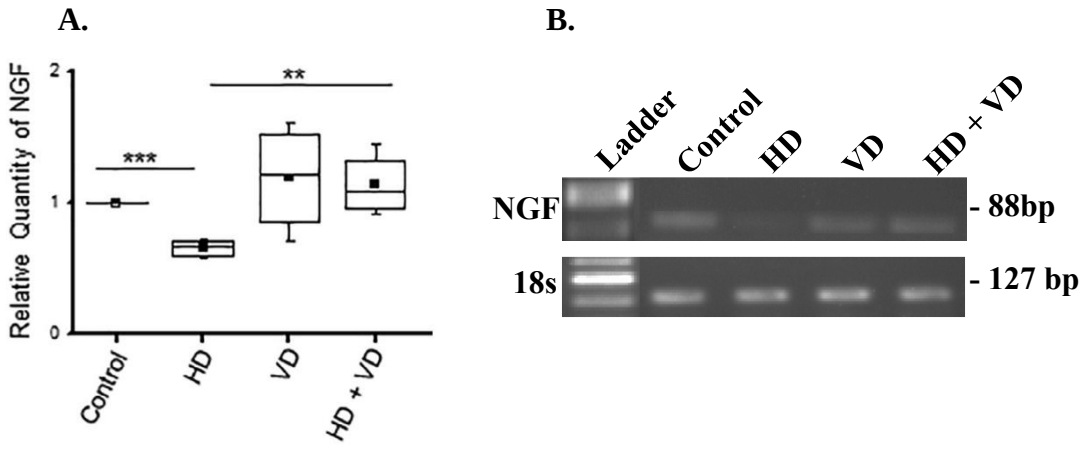

Fig. 22. mRNA expression of NGF from the striatal tissues of mice depicting neuroprotective effect of 500IU/kg of VD by semi-quantitative PCR. (A) VD administration rescued the mRNA expression of NGF in the striatum of 3-NP induced HD mice (Group IV vs Group II, n = 4, p = 0.001, paired sample t-test). NGF expression was significantly downregulated in HD mice as compared to control (Group II vs Group I, n = 4, p = 0.006, paired sample t-test). Representative gel images of PCR results for NGF. (B) Representative gel images of semi-quantitative PCR results for NGF. Data is represented as normalized mean ± SEM value against control.

3.3.1.2. VD supplementation increases the mRNA expression of brain derived neurotrophic factor in HD mice by real-time PCR:

Alterations in the mRNA expression of BDNF was analyzed in striatal tissues from all the four groups of mice by RT-PCR. RT-PCR results for BDNF expression in the striatum showed a significant change in the gene expression induced by VD supplementation in HD mice (n = 3, p = 0.04, Kruskal-Wallis test, **Fig. 23**). HD mice showed a significant decrease in the gene expression of BDNF as compared to Controls (Group II vs Group I; 0.53 ± 0.06 vs 1.00 ± 0.00, n = 3, p = 0.001, unpaired sample t-test). VD administration after 3-NP injection robustly increased the BDNF expression in Group IV mice (3.10 ± 0.57) when compared with HD mice (Group II mice; 0.53 ± 0.06, n = 3, p = 0.01, unpaired sample t-test, **Fig. 23**) reflecting that the biological effect of VD was not compromised by 3-NP induction. In addition, no significant difference in the BDNF expression was observed in the striatal tissues of Group III with respect to Group I mice

(VD vs Control; 1.41 ± 0.40 vs 1.00 ± 0.00, n = 3, p = 0.35, unpaired sample t-test, **Fig. 23**).

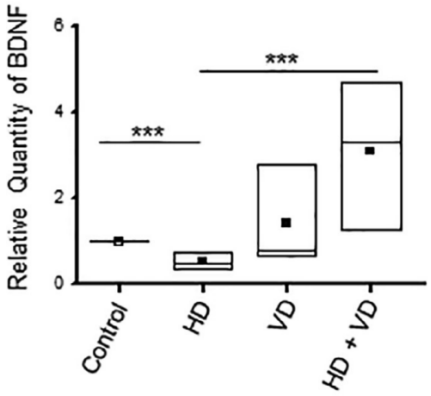

Fig. 23. RT-PCR results depicting robust enhancement in the mRNA expression of BDNF in the striatum of HD mice on VD administration (Group IV vs Group II, n = 3, p = 0.01, unpaired sample t-test. Striatal tissue of HD mice showed a significant decrease in the gene expression of BDNF (Group II vs Group I, n = 3, p = 0.001, unpaired sample t-test. Data is represented as normalized mean ± SEM value against control.

3.3.2. VD supplementation attenuates oxidative stress by regulating the gene expression of antioxidant markers:

To observe the effect of VD supplementation on the gene expressions of antioxidant markers, we performed semiquantitative PCR in all the four groups of mice (Group I to Group IV). mRNA expressions of Superoxide dismutase 1 (SOD1), Superoxide dismutase 2 (SOD2), Glutathione peroxidase 4 (GpX4), and Catalase (Cat) were subsequently analyzed.

3.3.2.1. VD did not show any effect on the gene expression of superoxide dismutase 1 and 2 in the striatum of HD mice:

The effect of VD supplementation did not significantly change the gene expression of superoxide dismutase 1 (SOD1) among the four groups of mice (p = 0.71, one-way ANOVA, **Fig. 24A and B**). Striatal tissue from HD mice showed no change in SOD1 mRNA expression (0.86 ± 0.42, n = 4) when compared with Group I (Control; 1.00 ± 0.00, n = 4, p = 0.38, paired sample t-test, **Fig. 24A**). VD administration in HD mice also showed no significant change in SOD1 expression in the striatal samples of Group IV mice (HD +

VD; 1.57 ± 0.45) when compared with Group II animals (HD; 0.86 ± 0.42, n = 4, p = 0.99, paired sample t-test, **Fig. 24A**). VD supplementation alone did not affect SOD1 mRNA expression in Group III mice when compared with Group I (VD vs Control; 1.41 ± 0.43 vs 1.00 ± 0.00, n = 4, p = 0.79, paired sample t-test, **Fig. 24A**).

Superoxide dismutase 2 (SOD2) mRNA expression also remained unchanged among all the four groups of mice either on 3-NP treatment or VD supplementation (p = 0.47, one-way ANOVA, **Fig. 24A**). SOD2 mRNA expression in HD mice was modulated by ~0.6 fold as compared to Control but did not reach significance (1.57 ± 0.35, n = 4, p = 0.90, paired sample t-test, **Fig. 24B**). Striatal samples from Group IV mice showed an insignificant change in SOD2 mRNA expression when compared with Group II mice (HD + VD vs HD; 0.99 ± 0.27 vs 1.57 ± 0.35, n = 4, p = 0.99, paired sample t-test, **Fig. 24B**). Also, no change in the expression of SOD2 was observe in Group III mice supplemented with only VD when compared with Group I mice (VD vs Control; 1.25 ± 0.37 vs 0.99 ± 0.01, n = 4, p = 0.54, paired sample t-test, **Fig. 24B**).

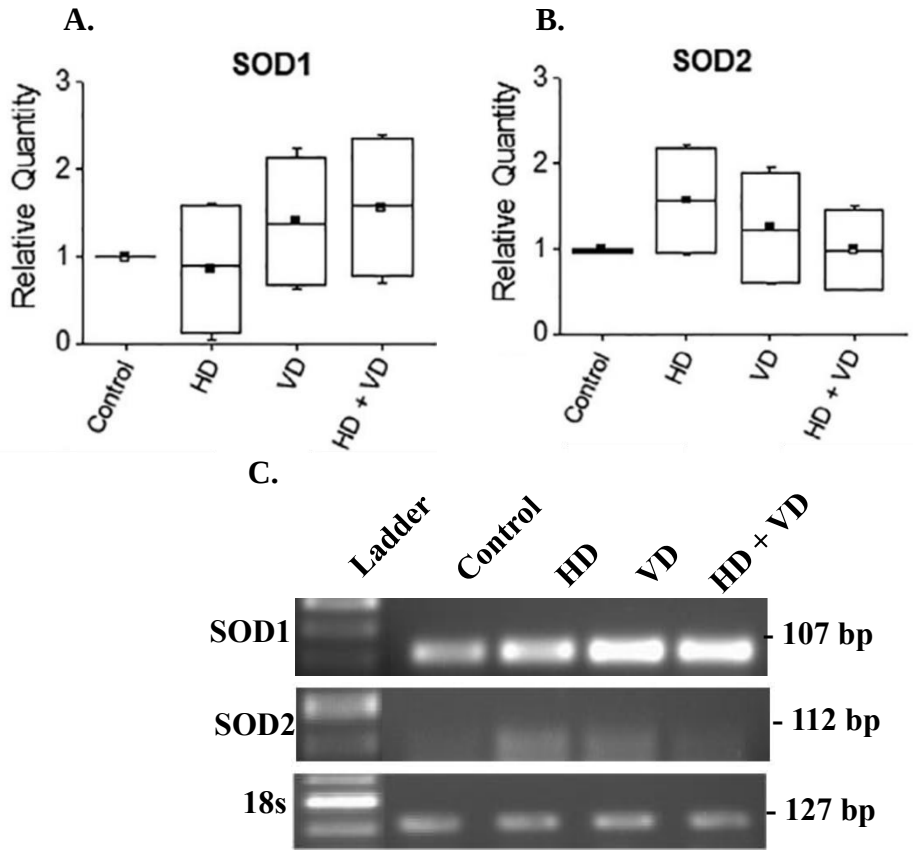

Fig. 24. mRNA expression of superoxide dismutase from the striatal tissues of mice depicting neuroprotective effect of 500IU/kg of VD. (A) On 30th day, no significant change in the mRNA expression of superoxide dismutase1 (SOD1) was observed across all groups of mice (n = 4, p = 0.71, one-way ANOVA). VD induction produced no change in the striatal expression of SOD1 in Group IV mice as compared to HD mice (Group IV vs Group II, n = 4, p = 0.99, paired sample t-test. (B) No significant change in the mRNA expression of superoxide dismutase2 (SOD2) was observed across all groups of mice (n = 4, p = 0.47, one-way ANOVA). VD supplementation did not significantly rescue the expression of SOD2 in Group IV mice as compared to HD mice (Group IV vs Group II, n = 4, p = 0.99, paired sample t-test). (C) Representative gel images of semi-quantitative PCR results for SOD1 and SOD2. Data is represented as normalized mean ± SEM value against control.

3.3.2.2. VD alleviates oxidative stress by decreasing the gene expression of glutathione peroxidase 4 in HD:

On the 30th day after 3-NP induction in HD mice, an overall change in the gene expression of glutathione peroxidase 4 (GpX4) in the striatal tissue was observed ($f_{(3)} = 14.06$, $p < 0.001$, one-way ANOVA, **Fig. 25**). PCR data for GpX4 revealed that 3-NP treatment caused a significant increase in the expression of GpX4 in the striatum of HD mice as compared with Group I mice (Group II vs Group I; 2.09 ± 0.22 vs 1.00 ± 0.00, n = 4, p = 0.008, paired sample t-test, **Fig. 25**). mRNA expression of GpX4 in Group IV mice (HD + VD) decreased with VD administration as compared to the HD mice (Group IV vs Group II; 1.19 ± 0.11 vs 2.09 ± 0.18, n = 4, p = 0.007, paired sample t-test, **Fig. 25**), to roughly control levels. Similarly, VD supplementation alone in Group III mice did not change GpX4 expression relative to Group I (VD vs Control; 1.08 ± 0.05 vs 1.00 ± 0.00, p = 0.99, paired sample t-test, **Fig. 25**).

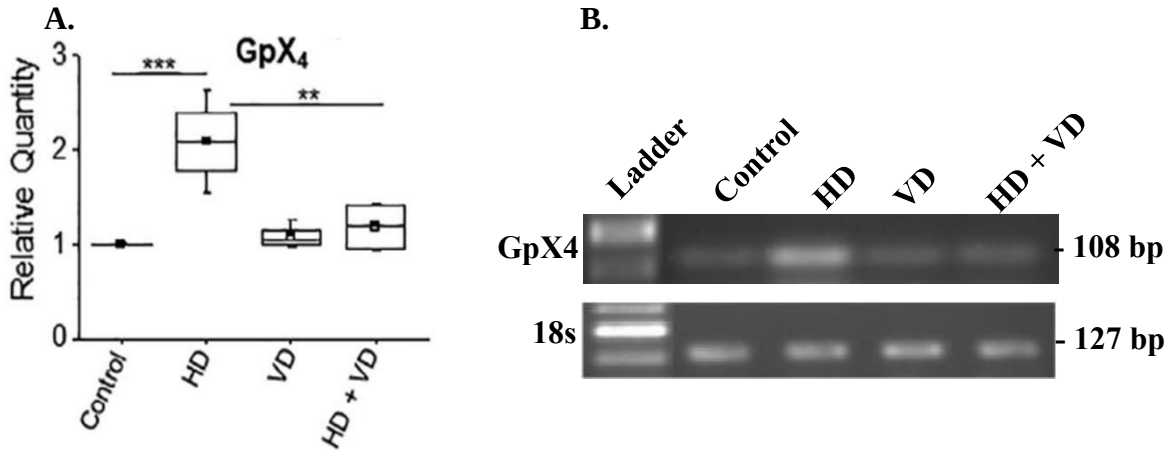

Fig.25. mRNA expression of glutathione peroxidase from the striatal tissues of mice depicting neuroprotective effect of 500IU/kg of VD. (A) Graph depicting enhanced expression of glutathione peroxidase on 3-NP injection in HD mice on 30th day which got substantially decreased on VD administration (n = 4, p < 0.001, one-way ANOVA; Group II vs Group I, n = 4, p = 0.008; Group IV vs Group II, n = 4, p = 0.007, paired sample t-test). (B) Representative gel images of semi-quantitative PCR results for GpX4. Data is represented as normalized mean ± SEM value against control.

3.3.2.3. Effect of VD on mRNA expression of catalase in HD:

Similar results were seen with expression of the antioxidant enzyme catalase (Cat). PCR data from the 30th day post-HD induction revealed an overall change in catalase expression across all the four treatment groups ($p < 0.001$, one-way ANOVA, **Fig. 26**). 3-NP injected HD mice showed a significant increase in the enzyme expression as compared with Group I mice (Group II vs Group I; 2.02 ± 0.18 vs 1.00 ± 0.00, n = 4, p = 0.005, paired sample t-test, **Fig. 26**). VD administration appears to reduce the oxidative stress in HD mice as seen by the decrease in catalase expression in Group IV mice (HD + VD) (Group IV vs Group II; 1.38 ± 0.03 vs 2.02 ± 0.18, n = 4, p = 0.02, paired sample t-test, **Fig. 26**). VD supplementation alone in Group III mice also showed a decrease in the mRNA expression of catalases when compared to HD mice but was not significant (VD vs HD; 1.72 ± 0.03 vs 2.02 ± 0.18, p = 0.08, paired sample t-test, **Fig. 26**). VD supplementation in Group IV mice (HD + VD) showed a decrease in the expression of antioxidants markers with a subsequent partial rescue in the body weight (**Fig. 21**).

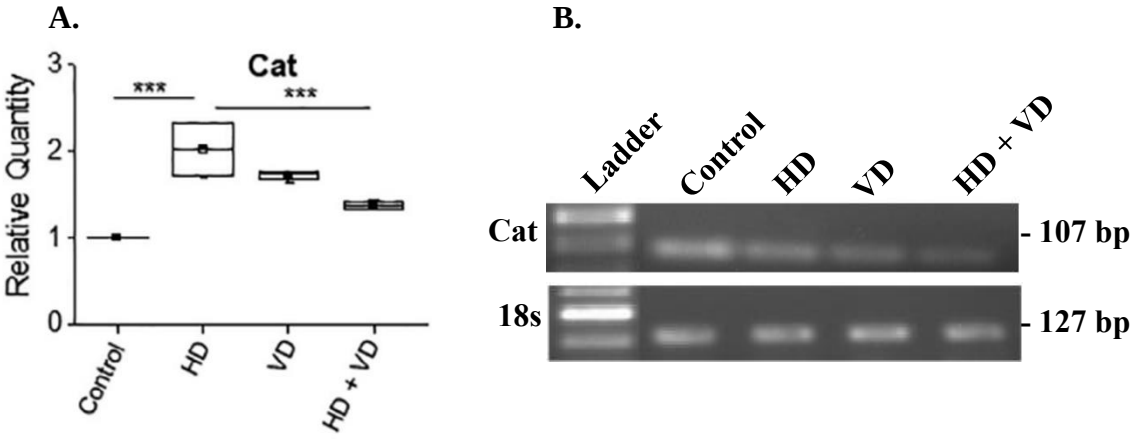

Fig. 26. mRNA expression of Catalase from the striatal tissues of mice depicting neuroprotective effect of 500IU/kg of VD. (A) Graph depicting rescue effect of VD on catalase expression in Group IV mice as compared to Group II (HD) (n = 4, $p < 0.001$, one-way ANOVA). Striatal expression of catalases was enhanced in HD mice (Group II vs Group I, n = 4, p = 0.005, paired sample t-test) which got diminished on VD induction in Group IV mice (n = 4, p = 0.02, paired sample t-test). (B) Representative gel images of semi-quantitative PCR results for Cat. Data is represented as normalized mean ± SEM value against control.

3.3.3. Restorative action of VD takes place through an increase in the protein expression of VDR in HD:

The effect of VD supplementation on expression of the VDR in the striatum was elucidated by western blot analysis ($f_{(3)} = 5.48$, $p = 0.01$, one-way ANOVA, **Fig. 27**). 3-NP mediated neurodegeneration caused a significant decrease in VDR expression by ~0.54 fold in HD mice (Group II) as compared to the Control (Group II vs Group I, 0.46 ± 0.15 vs 1.00 ± 0.00, n = 4, p = 0.02, paired sample t-test, **Fig. 27**). VD supplementation rescues this effect as Group IV mice (HD + VD) showed a significant increase in the expression of VDR by ~ 2-fold as compared to Group II (HD) mice (1.28 ± 0.26 vs 0.46 ± 0.15, n = 4; p = 0.04, paired sample t-test, **Fig. 27**). An enhancement in the protein expression of VDR was observed in Group III mice, supplemented with only VD as compared to Group I mice but did not reached significance (VD vs Control; 1.52 ± 0.25 vs 1.00 ± 0.00, n = 4; p = 0.13, paired sample t-test, **Fig. 27**). Our results parallel the finding of Lima and team where VD administration enhanced the expression of VDR in the hippocampus (Lima et al., 2018).

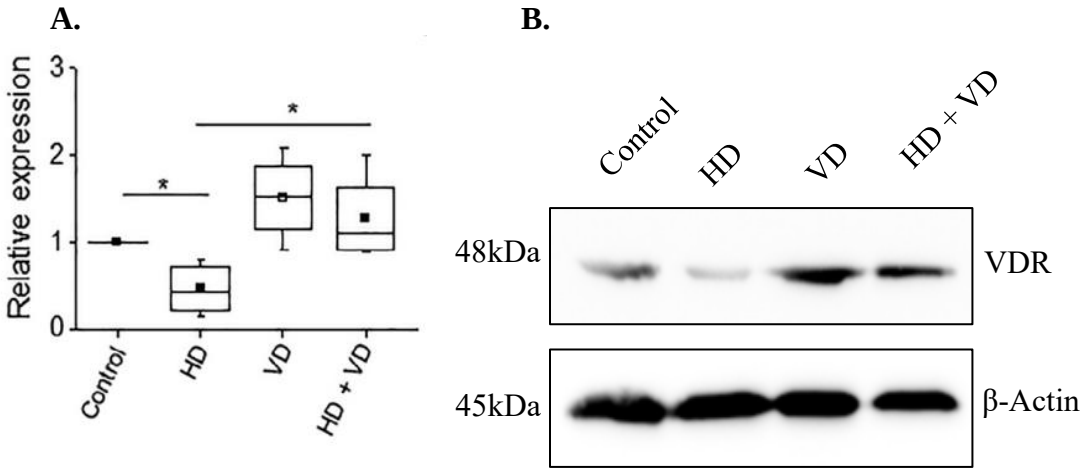

Fig. 27. Enhanced protein expression of VDR in the striatum of HD mice. (A) Graph depicting neuroprotective effect of 500IU/kg of VD in HD mice. The protein expression of VDR got significantly compromised in HD mice and reversed substantially on VD administration (n = 4, p = 0.01, one-way ANOVA; Group II vs Group I, n = 4, p = 0.02; Group IV vs Group II, n = 4, p = 0.04, paired sample t-test). (B) Blot representation to analyze the protein expression of VDR. Data is represented as normalized mean ± SEM value against control.

3.4. Discussion:

In the last decade, μg/kg VD or 1α,25-dihydroxyVitamin D3 and its analogues have been explored for their usefulness in brain disorders. A number of studies have reported a link between low serum level of VD in patients affected by neurodegenerative and neuropsychiatric disorders like AD, PD, HD, Schizophrenia, sleep disorders, autism, and depression (Bakhtiari-Dovvombaygi et al., 2021; Chabas et al., 2013; Kim et al., 2006; Koduah et al., 2017; Mohamed et al., 2015; Morello et al., 2018). Under a number of neuropathological conditions, VD supplementation has shown to have a myriad of biological functions including reducing the expression of oxidative stress markers and neuro-inflammatory markers and increasing the expression of neurotrophins (Latham et al., 2021; Lima et al., 2018; Mohamed et al., 2015; Rodrigues et al., 2019). Based on the study reported in chapter 2 that treating 3-NP HD model mice with 500IU/kg/day of VD produces significant improvements in movement and motor performance (**Fig. 17 and 19**). The dose of VD was chosen based on prior studies of its neuroprotective, antidepressant, and antioxidant effect in rodent model (Gueye et al., 2015; Kolla and Majagi, 2019; Mohamed et al., 2015). A study by Rodrigues and colleagues demonstrated that in rodent model of sporadic dementia of Alzheimer's type, 500IU/kg/day of VD was enough to reduce oxidative stress markers and restore cholinergic function by decreasing acetylcholine esterase activity in synaptosomes (Rodrigues et al., 2019).

The enhancement in locomotory and rotarod performances of HD mice post injected with VD (Group IV; HD + VD) was accompanied with an enhancement in the expression of brain derived neurotrophic factor (BDNF), nerve-growth factor (NGF), and the VDR (**Fig. 22, 23, and 27**). Previous studies have found that VD mediates an increase in the expression of VDR, tyrosine hydroxylase (TH), the dopamine transporter (DAT), and brain derived neurotrophic factors (BDNF) (Nimitphong et al., 2011). VD mediates its biological effect via VDR by acting as transcriptional regulator for some important neurotrophins in the brain like NGF and BDNF (Bayo-Olugbami et al., 2022; Johri et al., 2013; Nadimi et al., 2020; Taniura et al., 2006; Zuccato and Cattaneo, 2007). To test some of these previously reported targets, semi-quantitative PCR and RT-PCR was carried out to explore VD-induced gene expression of neurotrophins in Control and 3-NP treated group

of mice. In agreement with earlier literature reports, we found a significantly decreased expression of BDNF and NGF in 3-NP injected HD mice, but this profoundly augmented in the Group IV mice (HD + VD) with supplementation of VD (500IU/kg) (**Fig. 22 and 23**) (Allen et al., 2013; Livak and Schmittgen, 2001; Zuccato and Cattaneo, 2007). Numerous studies have highlighted the importance of neurotrophic factors like BDNF and NGF as potential therapeutics for neurodegenerative diseases such as AD, PD, and HD (Calvello et al., 2017; Lima et al., 2018; Mohamed et al., 2015; Rodrigues et al., 2019). In particular, *in-vivo* and *in-vitro* findings from Zuccato et al (2001) suggest that restoring BDNF production in cortical neurons during HD could restore the survival signal required by the dying striatal neurons (Zuccato et al., 2001; Zuccato and Cattaneo, 2007). The same study also provided evidence using genetic models of HD that mutant huntingtin profoundly diminished the cortical production of BDNF. Further, the work conducted by Navarro and team suggests BDNF to be the most effective factor in preventing the loss of striatal neurons in HD (Navarro et al., 2000). Our data demonstrate that the gene expression of BDNF and NGF was significantly compromised in 3-NP induced HD mice (Group II) and was substantially reversed upon VD administration in Group IV mice. This result suggests a direct therapeutic benefit of VD in combating 3-NP induced striatal neurodegeneration via BDNF and NGF in the striatum (**Fig. 22 and 23**). NGF and BDNF are established candidates for combating the death of neurons observed in a range of neurodegenerative disorders (Allen et al., 2013; Gil-Mohapel, 2012; Zuccato et al., 2001). VD supplementation possibly enhances the survival signals from neurotrophins to reduce neurodegeneration and combat striatal neuronal loss as observed in the rat model of AD (Mohamed et al., 2015). These results indicate that VD could alleviate behavior deficits in 3-NP induced HD mice via enhancement in neurotrophins expression in the striatum.

The enhancement in the production of neurotrophins like BDNF could act to reduce oxidative stress in neurodegenerative diseases including HD (Allen et al., 2013; Paul and Snyder, 2019). Oxidative stress markers allow assessment of the status of the biological samples where it measures the capacity of the system to scavenge free radicals. To control the intracellular redox balance, cells have evolved a highly complex ROS scavenging network. Previous studies on the antioxidant role of VD have been controversial as some studies did not support an antioxidant function for VD and other studies observed an up-

regulation of the antioxidant markers (Ahmed et al., 2020; Bayo-Olugbami et al., 2022; Lima et al., 2018). To determine whether, in our model, similar pathways are activated we checked different antioxidant enzymes marker genes. The glutathione (GSH)-dependent enzymatic system is one of most important ROS balancing units that regulates cell survival against oxidative damage. GSH contributes to the maintenance of the intracellular redox environment either by disulfide-exchange reactions with oxidized proteins or by acting as a reducing agent for glutathione peroxidases. Out of seven Glutathione peroxidases of mammals, GpX4 is particularly important due to its critical role in determining the cell membrane redox state. Increased expression of GpX4 indicates lipid based oxidative stress (Tagliaferri et al., 2019). In Group II (HD animals) we found a significant increase in expression of GpX4 indicating higher oxidative stress and this was attenuated upon supplementation with VD (Group IV, **Fig. 25**). Catalase is one of the crucial antioxidant enzymes that mitigates oxidative stress by destroying cellular hydrogen peroxide to produce water and oxygen (Tagliaferri et al., 2019). Supporting the GpX4 expression data which indicates higher oxidative stress, HD (Group II) animals showed increased expression of catalase, which was again diminished by VD supplementation. This suggests that VD supplementation reduces oxidative stress and leading to the subsequent downregulation of antioxidant enzymes. We could not find the significant differences in SOD1 and SOD2 expression possibly because its activation depends on very specific ROS species.

The antioxidant effect of VD supplementation in HD mice was accompanied by enhancement in the protein expression of VDR in the striatum (**Fig. 27**). Previous studies have reported that the biological activity of VD happens via upregulation of VDR in other neurodegenerative diseases like AD, PD, stress etc. Therefore, the protein expression of VDR was analyzed in Group IV mice (HD + VD) pre-injected with 3-NP. On the 30^{th} day, a robust expression of VDR by ~2 fold was observed in HD mice supplemented with VD (**Fig. 27**). HD mice (Group II) showed a significant decrease by ~0.54 fold in the VDR expression as compared to Control (Group I). This enhanced VDR expression could help in attenuating the toxic effect of 3-NP thereby reducing antioxidant stress markers and increasing neurotrophins expression in Group IV mice. The improvement in motor performance observed in HD mice could also occur due to increased VDR signaling at the

neuromuscular junction as seen previously (Lima et al., 2018). Previous studies have demonstrated that VDR signaling alleviates oxidative stress and increases production of neurotrophins like BDNF (Bakhtiari-Dovvombaygi et al., 2021; Nadimi et al., 2020). It is likely that in our study, the rescue effect of VD observed in behavior tasks involves the VD-VDR signal transduction pathway, potentiating survival signals via neurotrophins and decreasing oxidative stress, which in turn downregulates antioxidant stress markers (**Fig.22, 23, 25 and 26**). It is known that VDR signaling is vital for mitochondrial integrity, combats ER stress and strengthens skeletal muscle activity at neuromuscular junction (Bakhtiari-Dovvombaygi et al., 2021; Maity et al., 2022). In summary, our data suggests that VD mediates a neuroprotective effect in the striatum via enhancement in the expression of VDR and vital neurotrophins, like BDNF and NGF, crucial for survival signals in HD.

Chapter 4

Unprecedented effect of Vitamin D3 on T-cell receptor beta subunit and alpha7 nicotinic acetylcholine receptor expression in 3-nitropropionic acid induced mouse model of Huntington's disease

4.1. Introduction:

One of the breakthroughs in the field of immune-neuronal interaction came 35 years ago when neuroscientists discovered the neuronal role of cytokine, interleukin-1 (IL-1) in the modulation of neurotransmitters release and explored its contribution toward immune-brain interaction (Kabiersch et al., 1988; Spadaro and Dunn, 1990). Thereafter, rapid advances were made in discovering the expression of immune molecules and receptors in the brain originally thought to be expressed only in the immune system. Immune proteins like major histocompatibility complex – I (MHC-I), β2 microglobulin (a co-subunit of MHC-I), and its potential binding partner CD3ζ (a protein complexed to receptors for MHC-I) were found to be expressed in neurons (Baudouin et al., 2008; Shatz, 2009; Komal et al., 2022). In addition to MHC-I, a study undertaken by Komal et al., 2014 reflected a possible effect of T-cell receptor activation (TCR) on α7 nicotinic acetylcholine receptor expression and function in the murine cortex. However, how immune resident protein, T-cell receptor beta subunit (TCR-β) expression in the central nervous system gets modulated under a neuropathological condition like those observed in Huntington's disease (HD) remains unexplored.

HD is a progressive, fatal, neurodegenerative disorder characterized by neuronal loss predominantly in the striatum, followed by the cortical region of the brain (Gil and Rego, 2008). Neuronal death results in motor, cognitive and working memory impairments typically associated with the disease pathology (Gil and Rego, 2008). Some of the neurotoxic conditions responsible for neuronal loss in the striatum and the cortex as seen in HD include enhanced neuroinflammation, increased oxidative stress, decreased neurotrophins production, and mitochondrial dysfunction (Cherubini et al., 2020; Maity et al., 2022; Rekatsina et al., 2020; Zuccato and Cattaneo, 2007). 3-NP induction in mice causes selective neuronal degeneration in the caudate and putamen of basal ganglia circuitry and recapitulates a wide range of neuropathological symptoms of HD (Brouillet, 2014). 3-NP is an irreversible inhibitor of succinate dehydrogenase and is a well-known toxin-induced model of HD (Kim et al., 2003). 3-NP injections in rodents have also been shown to cause neuroinflammation and neurochemical alteration due to increased oxidative stress (Ahuja et al., 2008). In this regard, an antioxidant effect of Vitamin D3 (VD;

cholecalciferol) at a dose of 500IU/kg/day was recently shown to significantly rescue motor dysfunction in a 3-NP-induced mouse model of HD (Manjari et al., 2022). VD administration also caused an enhancement in the gene expression of neurotrophins like nerve-growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in the striatum (Manjari et al., 2022).

There are shreds of evidence that VD mediates its biological effect by binding with the VDR and combats neuronal loss across a range of neuropsychiatric illnesses (AlJohri et al., 2019; Bakhtiari-Dovvombaygi et al., 2021; Buell and Dawson-Hughes, 2008; Chabas et al., 2013; Nimitphong and Holick, 2011; Rodrigues et al., 2019). Nonetheless, under such neuropathological conditions, as observed across a multitude of neurological disorders like AD, PD, Schizophrenia, and HD, impairment in cholinergic neurotransmissions are also discovered where specific activation of α7 nicotinic acetylcholine receptors (α7 nAChRs) have been shown to exhibit neuroprotective benefits (Caton et al., 2020; Egea et al., 2015; El Nebrisi et al., 2020; Foucault - Fruchard et al., 2017; Foucault-Fruchard et al., 2018; Hoskin et al., 2019; Marder, 2016; Quik et al., 2015; Tata et al., 2014; Zhao et al., 2021). However, the impact of VD supplementation on the neuronal gene expression of TCR-β subunit receptor and α7 nAChRs in HD remains largely unexplored. Also, 3-NP mediated increase in oxidative stress and its effect on acetylcholinesterase (AChE) activity in HD remains to be elucidated.

In the present study, we show that VD administration in HD mice preinjected with 3-NP significantly decreases the gene expression of TCR-β immune receptor and antioxidants like catalase (Cat), and glutathione peroxidase (GpX4) together with a concomitant reduction in the acetylcholinesterase activity in the cortex and striatal brain regions. No significant difference was observed between Group I (control mice) and Group III (mice supplemented only with VD), further supporting the present hypothesis that VD neuroprotective benefits were observed only when neurons were subjected to neurodegeneration on 3-NP administration. Overall in the present work, we primarily show an anticholinesterase activity of VD and its positive effect on α7 nicotinic acetylcholine receptor mRNA and protein expression together with a detrimental effect on the gene expression of the TCR-β subunit in HD.

4.2. Materials and methods:

4.2.1. Animal Procurement:

Ten to twelve weeks old male C57BL/6 mice (average weight; 26 ± 3 g) were acquired from Sainath Agencies, Hyderabad, India. Animals were group-housed (2 mice per cage) with *ad libitum* access to food and water. They were kept in a 12 h light/12 h dark cycle at 25±2 °C. All the animal experiments were carried out with the approval of the institutional animal ethics committee (IAEC), BITS - Pilani, Hyderabad (BITS/Hyd/IAEC/2019/10, BITS/Hyd/IAEC/2020/20). All efforts were made to minimize the number of animals used and their suffering.

4.2.2. Study design:

All the animals were acclimatized for two weeks and were then randomly divided into 4 experimental groups (Group I to Group IV). Intraperitoneal injections (i.p) of 3-nitropropionic acid (3-NP) and/or VD were given as described previously (Manjari et al., 2022). Briefly, 3-NP was given thrice at a dose of 25 mg/kg, every 12 h, for a total cumulative dose of 75 mg/kg. Intraperitoneal injections (i.p) of VD were undertaken at a dose of 500IU/kg/day from day 1 to day 15 (Manjari et al., 2022).

4.2.3. Experimental design:

The mice were randomly divided into four experimental groups for biochemical assays (**Table 5**).

i. **Group I:** Control group mice (C57BL/6) injected with 1X saline.

ii. **Group II:** 3-NP induced mice by i.p. injection (3-NP; 75 mg/kg) without VD-treatment (HD).

iii. **Group III:** Mice injected solely with 500IU/kg/day of VD for 15 days**.**

iv. **Group IV:** Post-intraperitoneal injection of 500IU/kg/day of VD to 3-NP (75 mg/kg) pre-injected mice for 15 days (HD + VD).

4.2.4. Drugs and reagents:

4.2.4.1 Cholecalciferol:

Cholecalciferol (Vitamin D3; VD) was purchased from Sigma-Aldrich, India (Cat No: C9756) and dissolved in 1% ethanol (diluted with sterile saline) on the day of injection (Mohamed et al., 2015). Mice were administered with 500IU/kg (12.5µg/kg/day) i.p. of VD as reported in chapter 2. VD administration was undertaken in Group III mice (only VD) and Group IV mice (HD+VD).

4.2.4.2. 3-Nitropropionic acid:

3-nitropropionic acid (3-NP) was purchased from Sigma-Aldrich, India (Cat No: N22908). Stock solutions of 3-NP (3 mg/ml) were prepared in 0.1M phosphate-buffered saline solution and were injected intraperitoneally at 25 mg/kg (3-NP; a cumulative dose of 75 mg/kg) thrice at 12 h intervals to respective groups of mice as described in chapter 2. Controls were treated with three doses of 1X saline at 12 h intervals.

4.2.5. RNA isolation and cDNA synthesis:

On the 30th day, mice from all four groups (i.e Group I to Group IV) were anesthetized using isoflurane (Rx, NoB506) and immediately decapitated for the extraction of cortical and striatal brain tissue samples. The respective brain tissue sample was placed into 1 ml of RNAiso PLUS (Takara Bio), sonicated on ice, and centrifuged after the addition of 200µl of chloroform for 30 minutes at 12,000g at 4°C (Eppendorf Refrigerated centrifuge, 542R). The isolation of the aqueous phase was followed by the addition of an equal volume of isopropanol (Hi-Media Laboratories, Molecular biology grade, India), followed by overnight incubation at -20°C. Sample washing was preceded with centrifugation at 12,000 g for 30 minutes at 4°C, followed by washing with 70% ice-cold ethanol. The obtained pellet was resuspended in nuclease-free water. DNase-treated samples (EN052, Thermo Scientific^{TM}, USA) were made up to 400µl using nuclease-free water, followed by sample purification using 1/10th volume of 3M sodium acetate and 2X volume of phenol: chloroform: isoamyl alcohol (Sisco Research Laboratories Pvt. Ltd., India) and centrifuged for 2 minutes at maximum speed at 4°C. The total concentration of purified RNA was estimated by the Nanodrop spectrophotometer (Nanodrop, Thermo

Fisher Scientific, USA). An equal amount of RNA from each group was used to reverse transcribe complementary DNA (cDNA) with the help of the Verso cDNA synthesis kit (Cat No: AB1453A, Thermo Scientific^{TM}, USA) as per the manufacturer's instruction. Briefly, 500 ng of purified RNA was taken from each group for cDNA synthesis with the following reaction conditions: 42°C for 1 h followed by 95°C for 2 minutes. The obtained cDNA was used for real-time polymerase chain reaction (RT-PCR). The expression of targeted genes was normalized to 18S RNA. All primers are listed in Table 7.

4.2.6. Analysis of gene expression for T-cell receptor alpha, T-cell receptor beta, tumor necrosis factor-alpha, interleukin 6, alpha7 nicotinic acetylcholine receptor, nuclear factor-kappa B and antioxidants by Real-time PCR:

The sequences of the immune receptor, TCR-alpha (TCR-α), TCR-beta (TCR-β), α7 nicotinic acetylcholine receptor (α7 nAChRs), nuclear factor-kappa B (NF-κB), pro-inflammatory cytokines (tumor necrosis factor-alpha (TNF-α) and interleukin 6 (IL-6)) and antioxidant marker genes (catalase (Cat) and glutathione peroxidase 4 (GpX4)) of the mouse genome were obtained from NCBI. The sequences were deposited in the IDT primer quest tool to get the most suitable primer for gene analysis. For antioxidant marker genes, catalases (Cat) and glutathione peroxidase 4 (GpX4) expression was undertaken in the study. All the genes, primer sequences, and amplicon sizes are listed in Table 1. The gene expression among the four groups of mice was assessed by RT-PCR in a CFX96 Touch Real-time PCR system (BioRad) using the GoTaq qPCR SYBR master mix (Cat No #A6001, Promega Corporation). The reaction mixture was prepared according to the manufacturer's protocol using ~12 ng of the cDNA template. Relative gene expression was quantified using the ΔCT method with respective primers (**Table 7**) and normalized to *18s* (forward 5'-ACGGAAGGGCACCACCAGGA-3'; reverse 5'-CACCACCACCCACGGAATCG-3'). We used the ΔΔCT method to determine the fold changes in the expression of TCR-β, α7 nAChRs**,** and oxidative stress markers (Livak and Schmittgen, 2001). Briefly, the threshold cycle (Ct) was extracted using Bio-Rad CFX Manager 3.1 software, and relative gene expression was calculated as represented in chapter 3.

Table 7. List of primers used in real time PCR studies to analyze the mRNA expression of different genes

Gene	Orientation	Sequence of primers (5' to 3')	Amplicon size
18s	Forward	ACGGAAGGGCACCACCAGGA	127
	Reverse	CACCACCACCCACGGAATCG	
TCR-α	Forward	CAAGTGACCCTTTCAGAAGATGA	106
	Reverse	GTGGACCTTGTCCAGGATATTG	
TCR- β	Forward	GTGAATGGCAAGGAGGTCCA	111
	Reverse	CCAGAAGGTAGCAGAGACCC	
GPx4	Forward	GCCCAATACCACAACAGTAGA	108
	Reverse	CCTGAACCACAGCGATGAA	
Cat	Forward	AATTGCCTCCACACCTTCAC	107
	Reverse	TCACCAAGCTGCTCATCAAC	
α7 nAChRs	Forward	GTACAAGGAGCTGGTCAAGAA	94
	Reverse	CAGGAGACTCAGGGAGAAGTA	
TNF-α	Forward	CTACCTTGTTGCCTCCTCTTT	116
	Reverse	GAGCAGAGGTTCAGTGATGTAG	
IL6	Forward	GGGATGTCTGTAGCTCATTCTG	101
	Reverse	AACTGGATGGAAGTCTCTTGC	
NF-κB	Forward	GGAACAGGTGGGATGTTGCT	187
	Reverse	GACTAAACTCCCCCTGATTCTGAAG	

4.2.7. Acetylcholinesterase activity assay:

The acetylcholinesterase (AChE) activity was assayed using Amplex® Red Acetylcholine/Acetylcholinesterase Kit (A-12217; Invitrogen) essentially following instructions as directed by the manufacturer. In the assay, AChE activity was assessed indirectly with the help of Amplex Red reagent, a highly sensitive dye for horseradish peroxidase (HRP). In the initial step, AChE transforms acetylcholine into choline and acts as a substrate for the choline oxidase enzyme that converts choline to betaine and H_2O_2. Following this step, H_2O_2 reacted at a ratio of 1:1 with Amplex red to produce the fluorescent product resorufin, which in turn was measured using a fluorescent plate reader (Spiromax, USA). To analyze AChE activity, the reaction was initiated using a 100µL working solution (50 µM acetylcholine, 200µM Amplex Red reagent, 0.1 U/mL choline oxidase, and 1 U/mL horseradish peroxidase [HRP]) which was added to 100 µl of lysate of the brain tissue sample from each respective group of mice. After 30 minutes of incubation at room temperature, the fluorescence intensity was measured at 590 nm emission wavelengths when excited at 560nm. The enzyme activity was calculated using AChE standard curve and data is represented as mU/mg protein after subtraction of the background fluorescent value for each sample fluorescent value.

4.2.8. Protein expression analysis of alpha7 nicotinic acetylcholine receptor by western blotting:

On the 30th day, cortical and striatal brain tissue was extracted from all four groups of mice. The tissue was homogenized in the lysis buffer (150 mM sodium chloride, 1.0% TritonX-100, 0.5% sodium dodecyl sulfate, and 50 mM Tris, pH 8.0). The protein concentration was determined using a Bradford protein assay kit (Bio-Rad, USA). We loaded equal amounts of protein (50 µg) run in a 12% gel, and then transferred to PVDF (Pall Corporation) membrane through a trans blot wet transfer system (Bio-Rad). The membrane was blocked using 5% BSA and incubated with respective primary and secondary antibodies for α7 nAChRs mouse mAb (CHRNA7, 1:500, #MA5-31691, Thermo Fischer); Anti-mouse IgG-HRP-linked antibody (1:5000, AB_10015289, Jackson ImmunoResearch Laboratories). Membranes stained with ponceau (ML045, Himedia) were used as a control for normalization. The signal intensities of the bands were captured

using the fusion pulse gel documentation system (Eppendorf, USA). ImageJ software was used to quantify the band intensities.

4.2.9. Statistical analysis:

Experimental data are represented as normalized values w.r.t to control. Data in the figures are represented by bar plots with mean data and standard error. to illustrate the distribution of normalized values for each respective group of mice (Group I to Group IV). Group data in the text are presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted using one-way ANOVA followed by either post hoc multiple pairwise analysis using Tukey's HSD tests or paired sample t-test. $p < 0.05$ was set as threshold of significance ($^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$). Statistical analysis of all data was performed using Origin 8.1.

4.3. Results:

4.3.1. VD supplementation decreases T cell receptor beta subunit expression in the cortex and striatum of HD mice:

To explore the chronic effect of VD on the immune receptor, T cell receptor beta (TCR-β) subunit mRNA expression, RT-PCR was performed on the cortical and striatal brain tissue samples extracted on the 30th day from all four groups of mice (Group I-Group IV). We found an overall significant change in TCR- β expression among all four groups of mice ($p = 0.004$; one-way ANOVA). HD mice (Group II) injected with a cumulative dose of 75mg/kg of 3-NP showed profound enhancement ~ 2-fold in the gene expression of the TCR-β subunit in the cortex when compared to that of control mice (Group II vs Group I; 3.16 ± 0.32 vs 1.00 ± 0.00, n = 6, $p = 0.009$, paired sample t-test; **Fig. 28A**). On the 30th day, post administration of 500IU/kg of VD in HD mice significantly subsided the gene expression of the immune receptor, TCR-β subunit in comparison to HD mice preinjected with only 3-NP (Group IV vs Group II; 1.06 ± 0.15 vs 3.16 ± 0.32, n = 6, $p = 0.02$, paired sample t-test; **Fig. 28A**).

Similarly, a comparable trend of the VD effect was observed from the striatal brain tissue samples of all four groups of mice ($p < 0.001$, one-way ANOVA). The expression of TCR-β in HD mice was upregulated by ~3-fold (3-NP) when compared to the control

(Group II vs Group I; 4.02 ± 0.52 vs 1.00 ± 0.00, n = 10, p = 0.005, paired sample t-test, **Fig. 28B**). VD supplementation significantly decreased the expression of TCR-β in the striatum of 3-NP injected mice (HD + VD) as compared with HD mice (Group IV vs Group II; 1.08 ± 0.07 vs 4.02 ± 0.52, n = 10, p = 0.008, paired sample t-test, **Fig. 28B**). Overall, these data represent that VD modulates the gene expression of the immune receptor, TCR-β under neuropathological conditions induced by 3-NP.

There is no significant effect of VD on the mRNA expression of TCR-α in both cortex (Group II vs Group I; 1.11 ± 0.12 vs 1.00 ± 0.00, n = 6, p = 0.4; Group IV vs Group II; 1.15 ± 0.12 vs 1.11 ± 0.12, n = 6, p = 0.86, paired sample t-test, **Fig. 28C**) and striatum (Group II vs Group I; 1.11 ± 0.08 vs 1.00 ± 0.00, n = 6, p = 0.005; Group IV vs Group II; 1.10 ± 0.12 vs 1.11 ± 0.08, n = 10, p = 0.008, paired sample t-test, **Fig. 28D**).

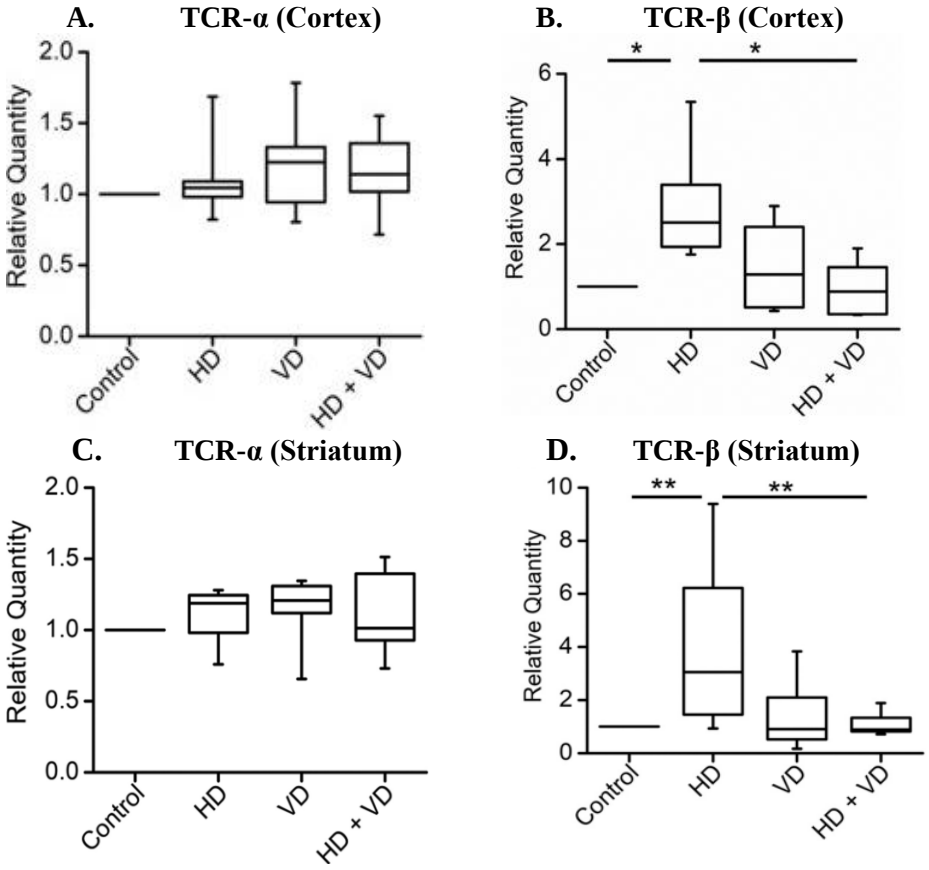

Fig.28. VD intake decreases the gene expression of the TCR-α and TCR-β subunit in the cortex and striatum of HD mice. (A) RT-PCR results didnot showed any significant in the cortical gene expression of the TCR-α among all four groups of mice (p=0.57, one-way ANOVA) (B) RT-PCR results demonstrated a significant increase in the cortical gene expression of the TCR-β subunit in Group II mice (HD vs control; n = 6, p = 0.009, paired sample t-test). VD administration to Group IV mice post-3-NP injection rescued the mRNA expression of the TCR-β subunit in the cortex of HD mice (HD + VD vs HD; n = 6, p = 0.02, paired sample t-test). (C) RT-PCR results didnot showed any significant in the striatal gene expression of the TCR-α among all four groups of mice (p=0.72, one-way ANOVA) (D) RT-PCR results depicting VD administration decreased the mRNA expression of the TCR-β subunit in the striatum of 3-NP-induced HD mice (HD + VD vs HD; n = 10, p = 0.008, paired sample t-test). TCR-β subunit expression was significantly upregulated in Group II as compared to Group I mice (HD vs control; n = 10, p = 0.005, paired sample t-test).

4.3.2. VD supplementation rescues the protein and mRNA expression of α7 nicotinic acetylcholine receptors in the cortex and striatum of HD mice:

The effect of VD supplementation on the protein expression of the α7 nicotinic acetylcholine receptor (α7 nAChRs) in the cortex was elucidated by western blot analysis. 3-NP mediated neurodegeneration caused a significant decrease in the α7 nAChRs protein expression in HD mice (Group II) as compared to the control (Group II vs Group I, 0.24 ± 0.08 vs 1.00 ± 0.00, n = 4, $p < 0.001$, paired sample t-test, **Fig. 29A**). VD supplementation rescued this effect as Group IV mice (HD + VD) showed an enhancement in the protein expression of α7 nAChRs as compared to Group II (HD) mice (1.13 ± 0.07 vs 0.24 ± 0.08, n = 4; $p < 0.001$, paired sample t-test, **Fig. 29A and C**). Real-time PCR analysis conducted on the striatal sample also showed a dramatic decrease in the mRNA expression of α7 nAChRs in HD mice which got rescued on VD administration (Group II vs Group IV 0.44 ± 0.01 vs 0.88 ± 0.10, n = 6, $p = 0.02$, paired sample t-test, **Fig. 29B**). α7 nAChRs mRNA expression got subsided in HD mice when compared to control mice (Group II vs Group I 0.44 ± 0.01 vs 1.00 ± 0.00, n = 6, $p < 0.001$, paired sample t-test, **Fig. 29B**). These results indicate that an increase in the gene expression of TCR-β (**Fig. 28**) was somehow causing a negative regulation of α7 nAChRs expression in HD and validates our previous finding where we showed that the entire octameric component of activated TCR downregulated the expression and function of the α7 nicotinic acetylcholine receptors (Komal et al., 2014). Here we show that neurotoxic conditions mimicked by 3-NP cause an increase in the gene expression of native immune proteins like TCR-β with concomitant downregulation in protein and mRNA expression of α7 nAChRs in the central nervous system.

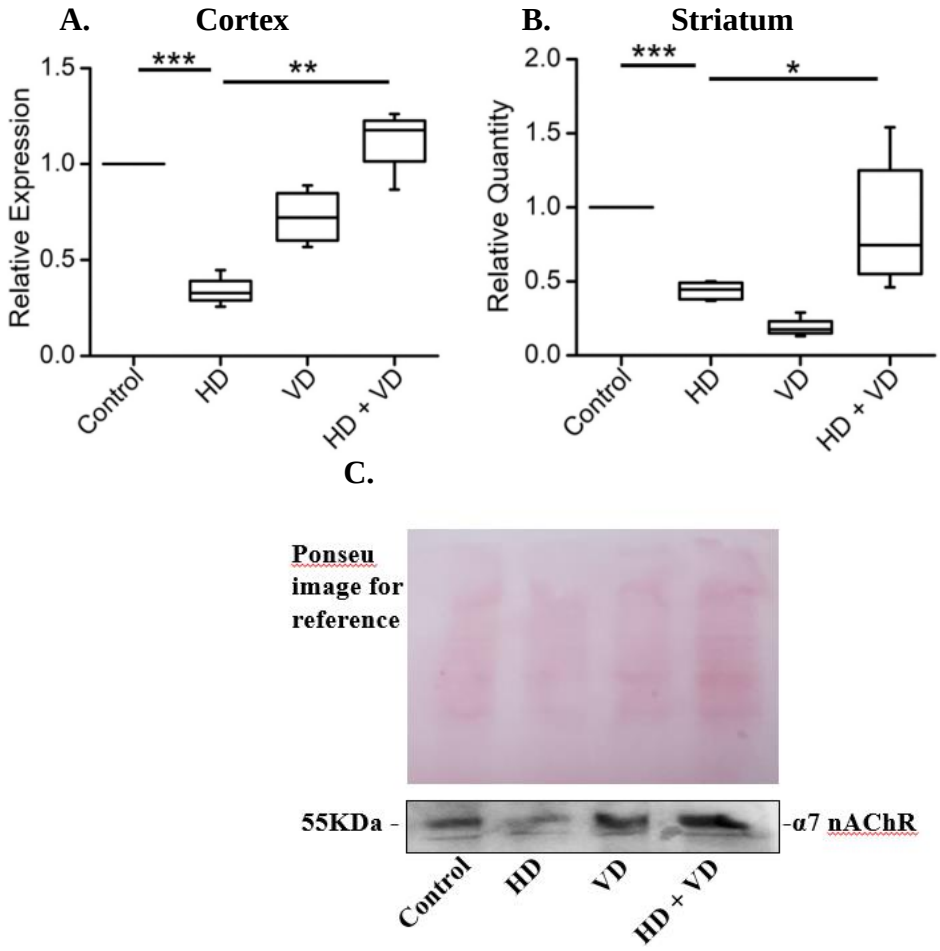

Fig.29. Effect of VD supplementation on the protein and gene expression of α7 nicotinic acetylcholine receptors (α7 nAChRs) in the cortex and striatum of HD mice. (A) On the 30th day, an overall change in the protein expression of α7 nAChRs was observed in cortical tissue samples from all the four groups of mice ($n = 4$, $p < 0.001$, one-way ANOVA). VD supplementation rescued the cortical expression of α7 nAChRs in Group IV mice (HD + VD) as compared to Group II (HD) mice ($n = 4$, $p < 0.001$, paired sample t-test). (B) A significant increase in the mRNA expression of α7 nAChRs was also observed in the striatal samples of 3-NP-induced HD mice (Group IV) on VD administration (HD + VD vs HD, $n = 6$, $p = 0.02$, paired sample t-test). The mRNA expression of α7 nAChRs got significantly decreased in Group II mice when compared with Group I mice (HD vs control; $n = 6$, $p < 0.001$, paired sample t-test). (C) Representative blot for protein expression of α7 nAChRs from the cortical tissues.

4.3.3. VD administration alleviates acetylcholinesterase levels in the cortex and striatum of HD mice:

To analyze the effect of VD on cholinergic neurotransmission, acetylcholinesterase (AChE) activity assay was performed on the cortical and striatal tissue samples from the respective four groups of mice. HD mice induced with 3-NP (75mg/kg) showed a significant rise in the AChE activity when compared with control mice (Group II vs Group I, 748 ± 70 mU/mg vs 417 ± 26 mU/mg, $n = 6$, $p < 0.001$, paired sample t-test, **Fig. 30A**), indicating the detrimental effect of 3-NP on cholinergic neurotransmission in the cortex. However, VD administration attenuated the effect of 3-NP and decreased the cortical AChE activity in HD mice (Group IV vs Group II; 502 ± 33 mU/mg vs 748 ± 70 mU/mg, $n = 6$, $p = 0.002$; paired sample t-test, **Fig. 30A**). A similar increase in AChE activity was also observed in the striatum of HD mice (Group II vs Group I, 49 ± 4 mU/mg vs 29 ± 3 mU/mg, $n = 8$, $p < 0.001$, paired sample t-test, **Fig. 30B**). On the 30th day, VD administration significantly attenuated the AChE activity in 3-NP- induced mice (HD + VD) when compared with HD mice group (Group IV vs Group II, 29 ± 3 mU/mg vs 49 ± 4 mU/mg, $n =8$, $p < 0.001$, paired sample t-test, **Fig. 30B).** These results indicate an anti-cholinesterase effect of VD in HD. The results of this study are in accordance with the previous findings where VD attenuated the AChE activity in the cerebral cortex of diabetic rats (Rodrigues et al., 2019). Thus, VD supplementation can rescue deficits in cholinergic neurotransmission by decreasing AChE activity and restoring acetylcholine (ACh) levels in HD.

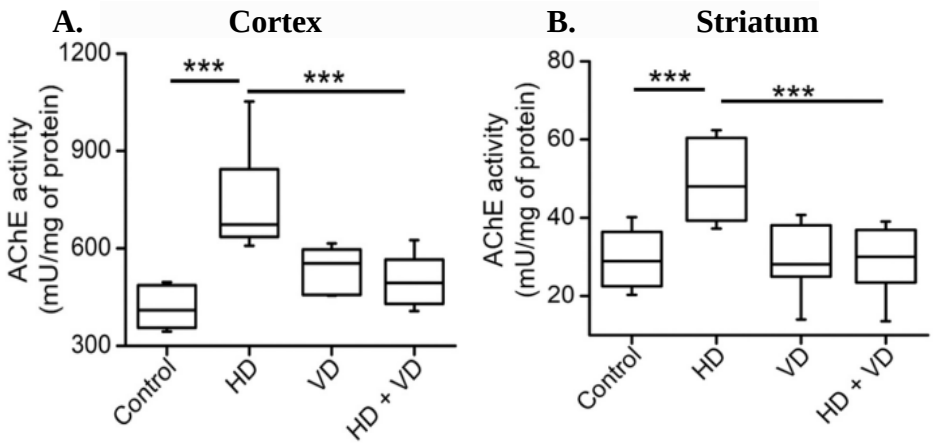

Fig. 30. Effect of VD supplementation on the enzymatic activity of acetylcholine esterase (AChE) in the cortex and striatum of HD mice (A) On the 30th day, a notable change in the activity of AChE was observed in the cortex of all four groups of mice ($n = 6$, $p < 0.001$, one-way ANOVA). VD induction significantly combated the activity of AChE in Group IV mice as compared to Group II (HD + VD vs HD; $n = 6$, $p = 0.002$, paired sample t-test). (B) A significant decrease in the activity of AChE was also observed in the striatal brain tissue samples of Group IV mice, supplemented with VD (HD + VD vs HD, $n = 8$, $p < 0.001$, paired sample t-test).

4.3.4. An Anti-inflammatory and anti-apoptotic effect of VD supplementation in HD mice:

A significant enhancement in the levels of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) is known to precede striatal neurodegeneration in HD (Chambon et al., 2023; Jia et al., 2022). To validate if 3-NP induction causes neuroinflammation in the striatum, we recorded the gene expression of vital neuroinflammatory markers like nuclear factor-kappa B (NF-κB) and proinflammatory cytokines like TNF-α and IL-6 from the striatal and cortical brain tissue samples from all the four groups of mice. HD mice injected with 3-NP showed a profound enhancement in the gene expression of NF-κB as compared to the control mice (Group II vs Group I; 7.42 ± 0.25 vs 1.00 ± 0.00, $n = 4$, $p < 0.001$, paired sample t-test, **Fig. 31A**). The mRNA levels of TNF-α (Group II vs Group I; 1.64 ± 0.06 vs 1.00 ± 0.00, $n = 4$, $p = 0.005$, paired sample t-test, **Fig. 31B**) and IL-6 in the striatum were also elevated on 3-NP induction (Group II vs Group I; 3.89 ± 0.50 vs 1.00 ± 0.00; $n = 4$, $p = 0.02$, paired sample t-test, **Fig. 31C**). Upon VD administration, the mRNA expression of NF-κB significantly subsided in HD mice (Group IV vs Group II; 0.57 ± 0.04 vs 7.42 ± 0.25, $n = 4$, $p < 0.001$,

paired sample t-test, **Fig. 31A**). VD intake by HD mice also showed a profound decrease in the mRNA expression of TNF-α (Group IV vs Group II; 1.04 ± 0.07 vs 1.64 ± 0.06, n = 4, p = 0.02, paired sample t-test, **Fig. 31B**) and IL-6 (Group IV vs Group II; 1.08 ± 0.13 vs 3.89 ± 0.50, n = 4, p = 0.01, paired sample t-test, **Fig. 31C**), reflecting its anti-inflammatory action.

A similar antagonistic effect of VD on inflammatory cytokines gene expression was observed in the cortex of HD mice. An increase in the cortical mRNA expression of TNF-α got substantially decreased in 3-NP-induced HD mice treated with VD (Group II vs Group I; 1.50 ± 0.07 vs 1.00 ± 0.00, n = 4, p = 0.01; Group IV vs Group II; 0.77 ± 0.03 vs 1.50 ± 0.07, n = 4, p = 0.002, paired sample t-test, **Fig. 31D**). Similarly, VD supplementation significantly decreased the mRNA expression of IL-6 in HD mice (Group IV vs Group II; 1.08 ± 0.07 vs 1.73 ± 0.14, n = 4, p = 0.01, paired sample t-test, **Fig. 31E**). Altogether, our data validate previous findings where HD pathogenesis was found to be associated with an aberrant NF-κB pathway activation (Khoshnan et al., 2004; Soylu-Kucharz et al., 2022).

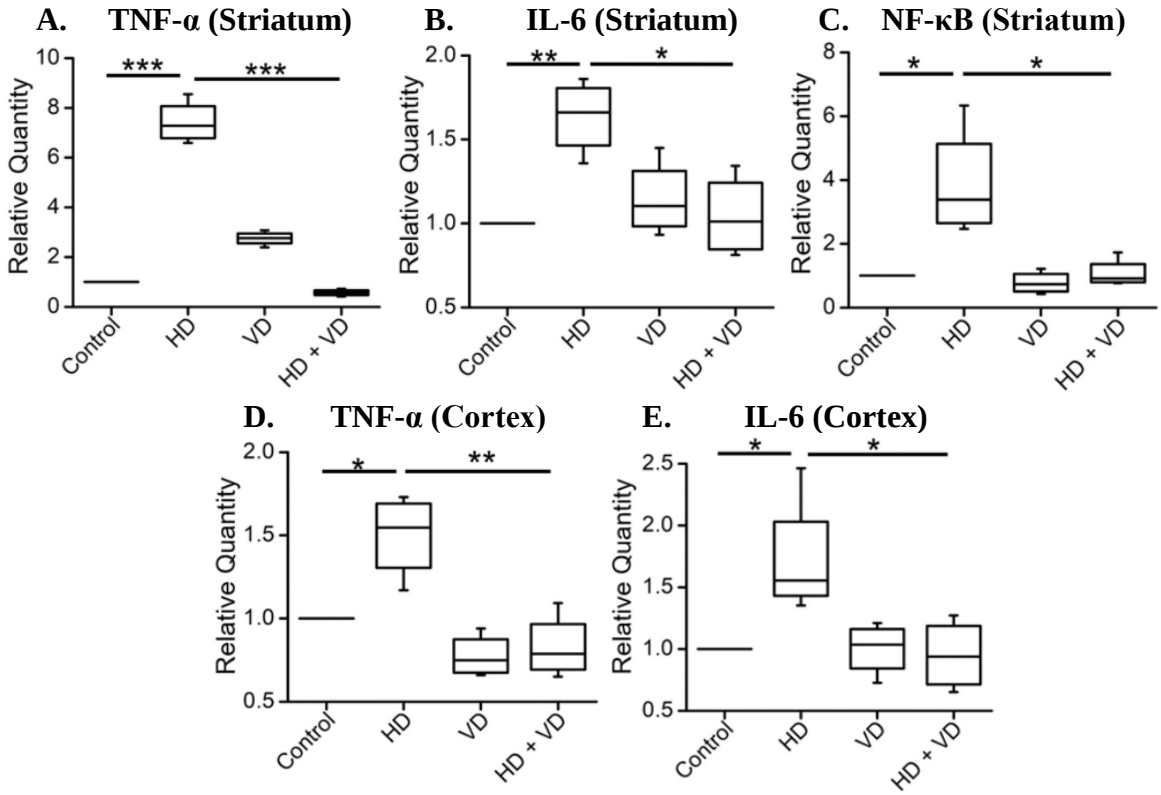

Fig.31. An Anti-inflammatory effect of 500IU/kg of VD in the striatum and cortex of 3-NP-induced HD mice. (A) Group IV mice administered with VD showed a significant reduction in the striatal gene expression of TNF-α (HD + VD vs HD, n = 4, p = 0.02, paired sample t-test) which got elevated in Group II mice injected with 75mg/kg of 3-NP (HD vs control, n = 4, p = 0.005, paired sample t-test). (B) An increased mRNA expression of another inflammatory cytokine, interleukin 6 (IL-6) was observed in the striatum of Group II mice (HD vs control, n = 4, p = 0.02, paired sample t-test) and was decreased in Group IV mice administered with VD (HD + VD vs HD, n = 4, p = 0.01, paired sample t-test. (C) mRNA expression of nuclear factor kappa B (NF-κB) was significantly increased in Group II mice (HD vs control, n = 4, p < 0.001, paired sample t-test) which got combated on VD supplementation (HD + VD vs HD, n = 4, p < 0.001, paired sample t-test). (D) A similar increase in the mRNA expression of tumor necrosis factor-α (TNF-α) was observed in the cortical brain tissue samples of Group II mice injected with 3-NP (HD vs control, n = 4, p = 0.01, paired sample t-test). The post-supplementation of VD for 15 days significantly attenuated the gene expression of TNF-α (HD + VD vs HD, n = 4, p = 0.002, paired sample t-test) and (E) interleukin 6 (IL-6) in the cortex of Group II mice (HD + VD vs HD, n = 4, p = 0.01, paired sample t-test). IL-6 gene expression was observed to be highly elevated in Group II mice on 3-NP injection (HD vs control, n = 4, p = 0.03, paired sample t-test).

4.3.5. VD administration in HD mice decreases oxidative stress as reflected by a reduction in key antioxidants gene marker expression in the cortex:

To observe the effect of VD supplementation on the gene expressions of antioxidant markers, we performed RT-PCR in all four groups of mice (Group I to Group IV). mRNA expressions of glutathione peroxidase 4 (GpX4), and catalase (Cat) were subsequently analyzed in the cortical brain samples.

4.3.5.1. Anti-oxidant effect of VD on the gene expression of glutathione peroxidase 4 in the cortex of HD mice:

On the 30th day after 3-NP-induction in HD mice, an overall change in the gene expression of GpX4 in the cortical tissues was observed ($n = 6$, $p < 0.001$, one-way ANOVA, **Fig. 32**). RT-PCR results of GpX4 revealed that 3-NP treatment elevated the gene expression of GpX4 in the murine cortex of HD mice as compared with control mice (Group II vs Group I; 2.83 ± 0.08 vs 1.00 ± 0.00, $n = 6$, $p < 0.001$, paired sample t-test, **Fig. 32**). mRNA expression of GpX4 in Group IV mice (HD + VD) got significantly decreased on VD administration as compared to the HD mice (Group IV vs Group II; 1.31 ± 0.14 vs 2.83 ± 0.08, $n = 6$, $p < 0.001$, paired sample t-test, **Fig. 32**).

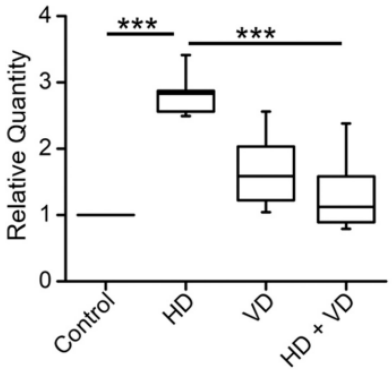

Fig. 32. VD administration rescues the gene expression of GpX4 in the cortex of HD mice. mRNA expression of glutathione peroxidase 4 (GpX4) was increased in Group II mice (HD vs control, $n = 6$, $p < 0.001$, paired sample t-test), which subsided on VD supplementation (HD + VD vs HD, $n = 6$, $p < 0.001$, paired sample t-test).

4.3.5.2. Effect of VD on the gene expression of catalase in the cortex of HD mice:

The effect of VD supplementation showed a remarkable change in the gene expression of catalase (Cat) among all four groups of mice in cortical samples (n = 8, p = 0.004, one-way ANOVA, **Fig. 33**). Cat mRNA expression was elevated in HD mice when compared with control mice (Group II vs Group I; 2.74 ± 0.33 vs 1.00 ± 0.00, n = 8, p = 0.008, paired sample t-test, **Fig. 33**). VD administration in HD mice combated the gene expression of catalases in the cortical samples of Group IV mice as compared with Group II mice (HD + VD vs HD; 1.29 ± 0.16 vs 2.74 ± 0.33, n = 8, p = 0.003, paired sample t-test, **Fig. 33**).

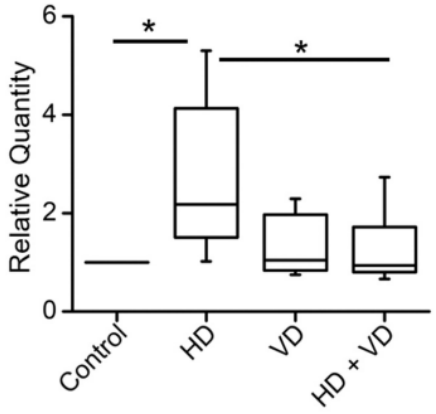

Fig. 33. VD administration rescues the gene expression of catalase in the cortex HD mice. The mRNA expression of catalase (Cat) was also found to be increased in the cortex of Group II mice as compared to Group I mice (HD vs control, n = 8, p = 0.008, paired sample t-test). The gene expression of Cat got alleviated on VD supplementation in Group IV mice reflecting its antioxidant effect (HD + VD vs HD, n = 8, p = 0.003, paired sample t-test).

4.4. Discussion:

The two primary pathological mechanisms commonly observed across all neurodegenerative diseases including HD are increased oxidative stress and neuroinflammation (Cherubini et al., 2020; Maity et al., 2022; Pérez-Rodríguez et al., 2020). Evidence indicates under these neurotoxic conditions there is an enhancement in the gene expression of the brain resident immune protein, the major histocompatibility complex-I (MHC-I, Wang et al., 2021; Welberg, 2013). Several studies also demonstrate that “immune receptors” like major histocompatibility complexes type I (MHC-I), the

cluster of differentiation-zeta (CD-3ζ), and leukocyte immunoglobulin-like receptor B2 (LILRB2) play a key role in neurodegenerative disorders and could be a potential therapeutic target for neurological disorders like Alzheimer's disease (AD) and Parkinson's disease (PD) (Kim et al., 2013; Welberg, 2013). However, the brain's resident T-cell receptor beta subunit's (TCR-β) gene expression modulation in a neurological disorder like HD is limited and remains largely unexplored. In this regard, studies have confirmed the neuroprotective capacity of VD in combating neuroinflammation, and oxidative stress, and restoring cholinergic signaling in different neurodegenerative disease models (Calvello et al., 2017; Koduah et al., 2017; Lima et al., 2018; Manjari et al 2022). A study by Rodrigues and colleagues specifically showed that VD upregulated VDR expression, restored oxidative damage, and decreased acetylcholinesterase (AChE) activity in a rodent model of AD. Our recent findings also highlighted the neuroprotective benefits of VD on motor dysfunction in 3-NP induced HD mice (Manjari et al., 2022).

In the present study, we demonstrate that chronic administration of 500IU/kg/day of VD (0-15 days) shows a long-lasting neuroprotective and anti-neurotoxic effect by decreasing the gene expression of the immune receptor, TCR-β subunit expression in both the cortex and striatum of HD mice (**Fig. 28B and D**). 3-NP administration is known to induce HD-like symptoms in rodents with a phenotype similar to the genetically inherited human disease (Brouillet, 2014; Brouillet et al., 2005). The striatal medium spiny neurons are more susceptible to neurotoxic conditions induced by 3-NP as compared to the cortical neurons (Singh et al., 2010). A significant increase in inflammatory mediators such as tumor necrosis factor-alpha (TNF-α) is also reported previously to be associated with the neurodegenerative effects of 3-NP in the striatum (Ahuja et al., 2008). Under such a neurotoxic environment, here we show that there is an increased gene expression of native immune proteins in the T-cell receptor- beta (TCR-β) subunit with no change in the gene expression of the T-cell receptor-alpha (TCR-α) subunit in the HD mice (**Fig. 28A and C**). We demonstrate that 3-NP-induced increased oxidative stress causes a profound enhancement in the gene expression of TCR-β in the murine cortex and striatum which gets subsided on VD administration (**Fig. 28B and D**). We also found that 3-NP mediated enhancement in a free radical generation, increased oxidative stress, and an increase in TCR-β subunit in HD mice was paralleled with an increase in acetylcholinesterase (AChE)

activity in the two brain regions most vulnerable to undergoing neuronal atrophy in HD i.e the cortex and the striatum (**Fig. 30A and B**). AChE is an important regulatory enzyme found in cholinergic neurons and its elevation indirectly reflects cholinergic dysfunction (Walczak-Nowicka and Herbet, 2021). Cholinergic deficiency and an increase in AChE levels have been shown previously to cause memory impairment in the 3-NP-induced rat model of HD (Menze et al., 2015). We show that VD administration decreases AChE activity in both the cortex and the striatum which also possibly reflects its importance as therapeutics to combat neuronal loss observed in this neurodegenerative disease (Vattakatuchery and Kurien, 2013; Walczak-Nowicka and Herbet, 2021). Much of the therapeutic potential of VD is reflected in the studies performed on AD, where the neuroprotective mechanism occurred via VDR signaling (Landel et al.,2016). In our recent finding also, we demonstrated that the striatal protein expression of VDR got rescued on post-VD supplementation (Manjari et al., 2022). Hence, it is very likely that VD-VDR mediated upregulation of neurotrophins like brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) activates the neuroprotective pathway in HD (Manjari et al., 2022).

In the past, T-cell receptor (TCR) activation has been shown to negatively regulate the expression and function of α7 nicotinic acetylcholine receptors in the murine frontal and prefrontal cortex (Komal et al., 2014). Also, previous studies have demonstrated that the α7 cholinergic receptor's activity is modulated via a variety of kinases like Protein kinase A (PKA) and Src-family kinases like Lck and Fyn kinase (Komal et al., 2015, 2014; Komal and Nashmi, 2015). The α7 nicotinic acetylcholine receptor comes under the family of ligand-gated ion channels where these ionotropic receptors are known to contribute toward cognition, attention, and working memory function which gets compromised in neurological disorders (Dau et al., 2013; Komal et al., 2011; Perutz et al., 1999; Suzuki et al., 2006; Vattakatuchery and Kurien, 2013). It is possible that under a neuropathological insult like those observed in HD, which is characterized by elevated neuroinflammation, apoptotic signals, and oxidative stress, an enhanced gene expression of the TCR- β subunit occurs with a concomitant downregulation in alpha 7 nicotinic acetylcholine receptors (α7 nAChRs) potentiating neuronal loss in the striatum.

VD supplementation rescued the protein and mRNA expression of α7 nAChRs and also restored the acetylcholine levels with a simultaneous reduction in the immune receptor, TCR-β subunit mRNA expression in the cortex, and the striatal brain tissue samples (**Fig. 28, 29 and 32**). In other words, restoration of cholinergic signaling in the striatum occurred with a downregulation in the gene expression of key proinflammatory cytokines like TNF-α and IL-6 in HD mice (**Fig. 31**). It is known that elevated levels of pro-inflammatory cytokines like TNF-α and NF-κB activity precede striatal neurodegeneration (Chambon et al., 2023; Khoshnan et al., 2004; Soylu-Kucharz et al., 2022). In our study, we show that VD intake by Group IV mice (HD + VD) showed a detrimental effect on NF-κB gene expression in the striatum (**Fig. 31A**). Thus, the anti-inflammatory and anti-apoptotic effect of VD reflects its neuroprotective benefits as observed previously across a wide range of neurogenerative diseases including HD (Buell and Dawson-Hughes, 2008; Calvello et al., 2017; Chabas et al., 2008; Lima et al., 2018; Manjari et al., 2022; Mohamed et al., 2015; Nimitphong and Holick, 2011; Rodrigues et al., 2019).

VD mediates its biological effect by interacting with the VDR (Landel et al.,2016). Hence, it is very likely that VD-VDR interaction mediates an anti-apoptotic signal by inhibiting the NF-κB mediated activation of vital pro-inflammatory cytokines gene expression and rescues cholinergic signaling deficits by combating AChE activity and restoring the expression of α7 nicotinic acetylcholine receptor in the cortex and the striatum. It may be argued that early intervention with VD can be proposed to have therapeutic benefits over a range of neurological disorders including HD possibly by downregulation of T-cell receptor-beta subunit expression (TCR-β) and inhibition of NF-κB mediated inflammatory cytokine pathway. The enhanced β subunit expression in the brain is justifiable in our findings as TCR-α subunit gene expression remained unchanged in all four groups of mice (**Fig. 28A and C**). However, we cannot rule out the possibility of invasion of peripheral T-lymphocytes invasion in our 3-NP mouse model of HD which also disrupts blood-brain barrier permeability (Kim et al., 2003), A functional anomaly of only the TCR-β subunit in neuropathological conditions is hypothesized in this work as recently proposed in our previous publication (Komal et al., 2022). It is speculated that striatal and cortical synapses may undergo enhanced synaptic pruning in HD via MHC-I

and TCR-β interaction under increased oxidative stress and mitochondrial dysfunction, which precedes the neurodegenerative processes observed across the plethora of neurodegenerative diseases (Komal et al., 2022).

This novel mechanism of downstream signaling cascade initiated by TCR-β in neurons may dictate the selective neurodegeneration of striatal and cortical neurons via downstream activation of kinase cascade and substantially abrogate the function and expression of nicotinic acetylcholine receptors under a neuropathological insult characterized by mitochondrial dysfunction, ER stress, elevation in oxidative stress, ATP depletion and increased cytokine storm as observed in HD and other neurological disorders (Komal et al., 2022). These statements merit additional research and future experiments will shed deeper insights into whether VD can interfere with the aberrant synaptic pruning preceding neurodegeneration in HD.

Chapter 5

General Discussion

The primary focus of the present study was to explore whether VD mediates a protective action on cortical and striatal neurons when subjected to neuropathological conditions like those observed in Huntington's disease (HD).

5.1. VD delays the progression of neurodegeneration in HD:

HD is an autosomal dominant neurodegenerative disorder characterized by impaired motor co-ordination, cognition, and behaviour (Gil-Mohapel, 2012). The main cause of HD is the unstable expansion of CAG repeats at the end of the Huntingtin (Htt) gene, which results in polyglutamate protein aggregates (Gil and Rego, 2008). When these aggregates interact with other cellular mechanisms, they cause mitochondrial dysfunction, oxidative stress, excitotoxicity, and inflammation (Blumenstock and Dudanova, 2020; Gil and Rego, 2008). Due to the increasing incidence of HD, researchers are focusing more and more on identifying therapies that can alter the course of HD in order to develop effective treatment options. The majority of therapeutic interventions focus on treating symptoms of motor, behavioral, and psychiatric disturbances (Ferguson et al., 2022; Mestre et al., 2009). A drug that is most commonly used by clinicians to treat HD patients is tetrabenazine (TBZ), which works by binding to the vesicular monoamine transporter (VAMT2) and depleting monoamines and dopamine from nerve terminals (Frank, 2010; Grigoriadis et al., 2017; Kenney et al., 2007). Despite the positive effects of this drug on chorea, there are notable side effects, including drowsiness (36.5%), parkinsonism (28.5%), depression (15.0%), insomnia (11.0%), nervousness or anxiety (10.3%), akathisia (9.5%) (Jankovic and Beach, 1997).

According to the previous study, we were able to find out that TBZ is a potential drug for HD that may be able to reduce the symptoms of HD, particularly chorea, which is the most common symptom of HD (Frank, 2010). As of now, it is unknown whether there is a possible medication that could delay the process of neurodegeneration in HD. Because there is a link between VD deficiency or insufficiency and neuronal atrophy in HD, we can propose VD as a therapeutic agent to combat or delay this neurological condition.

5.1.1. Intervention of age-related disorders with VD including HD:

Many of the hallmarks of aging are also seen in the mechanisms involved in the pathogenesis of Huntington's disease (HD; Berridge, 2017; Wilson et al., 2023). The activity of all these mechanisms declines with age and the process is more rapid in HD patients (Berridge, 2017). Although HD patients have the gene mutation from birth, it takes several decades for the mutation to manifest (Nopoulos, 2016). The hallmarks of aging and cellular alteration in HD have a striking overlap, therefore delaying biological aging could delay the onset or progression of HD symptoms (Berridge, 2017; Machiela et al., 2020). Hence, the role of aging in HD needs further investigation, and anti-aging therapies may show a beneficial effect on HD.

5.1.2. VD as a potential therapeutic agent for HD:

In the last two decades, a positive correlation is discovered between low blood serum levels of calcidiol and the risk of development of neurodegenerative diseases such as Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD, A. Lauer et al., 2020; Chai et al., 2019; Chel et al., 2013). In the case of HD, a study by Chel and collegues in year 2013 found a significant decrease in the serum levels of calcidiol in patients with HD when compared with controls (Chel et al., 2013). A rapid erosion in the levels of calcidiol is also documented in AD and PD patients. These neurological disorders are also associated with polymorphisms in the VDR which binds to its endogenous ligand, VD, and mediates its biological effects. In light of this, it can be speculated that a VD deficiency may play a role in the development of neurodegenerative diseases. All of these findings suggest that a deficiency or insufficiency of VD may be a risk factor that leads to the progression of neurodegeneration with advancing age. In concert with this idea, an experimental study carried out in the case of HD reflected an increase in life expectancy in a genetic rodent model of HD with 82 CAG repeats (Fort Molnár et al., 2016). In this study, however, the exact molecular mechanism that is responsible for the decrease in mortality was unknown (Fort Molnár et al., 2016). Our study provides convincing evidence that under neurotoxic conditions, VD supplementation can play a neuroprotective role in HD. VD administration in such neurological conditions boosts survival signals, combats oxidative stress, inflammation, apoptotic cell death, and increases the activity of

neurotrophins. It is very likely to propose that VD neuroprotective effect increases the life span of medium spiny neurons in the striatum by BDNF, VDR, and α7 nAChRs mediated signaling.

5.2. VD rescues spatial memory and behaviour phenotypes in HD:

The results from chapter 2 expand our understanding of the role of VD in regulating the behaviour and memory of HD mice. There has been significant evidence to prove our findings that VD alters behavior in age-related neurological diseases like AD and PD (Amende et al., 2005; Doncheva et al., 2022; Lima et al., 2018; Lin et al., 2022; Mohamed et al., 2015). The results of this thesis provide using exclusive evidence that 500IU/kg/day of VD supplementation can significantly improve locomotion, motor coordination, and spatial memory deficits of HD mice, administered with 3-NP) (Manjari et al., 2022). In this toxin-induced HD model, we have clearly shown that the 3-NP significantly mimics nearly all the hypokinetic symptoms as observed in HD. Our findings need further validation on the benefits of VD supplementation in a genetic model of HD.

5.3. VD administration upregulates VDR expression in HD:

In chapter 3 of this thesis, the protective role of VD was determined that the upregulation of VDR which in turn enhanced the expression of neurotrophins like brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the striatum and cortex of HD mice. The upregulation in the gene expression of BDNF and NGF boosts striatal neuronal survival. It is very likely to occur by activating the TrkB and TrkA receptors respectively (Wang et al., 2022). TrkB activation occurs via transphosphorylation of the src homolog domain (SH2) and phosphorylation of PLCγ and PI3K which further activated MAPK/ERK signaling. It is found that activation of MAPK signaling support neuronal survival, function, and synaptic plasticity as shown in the **Fig. 34**.

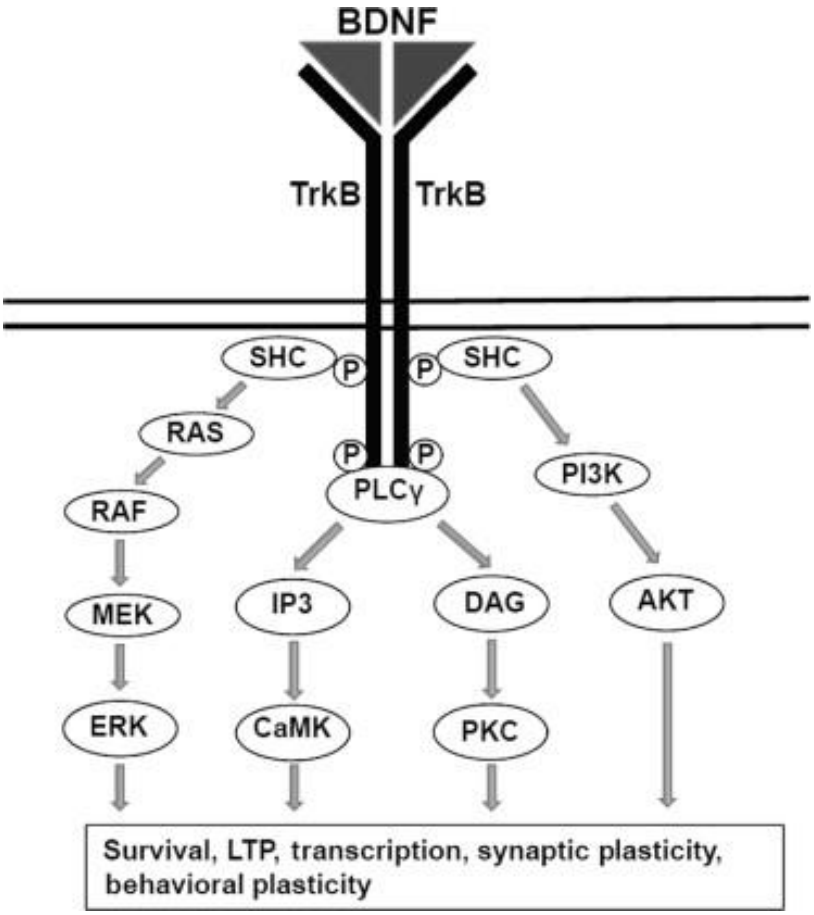

Fig. 34. Overview of BDNF signaling through TrkB receptors. Upon binding to BDNF, the receptor tyrosine kinase TrkB becomes phosphorylated. Phosphorylation at various sites leads to activation of downstream pathways. The PI3K pathway activates protein kinase B (AKT), leading to cell survival. The MEK and ERK pathway leads to cell growth and differentiation. The PLC pathway activates inositol trisphosphate (IP3) receptor to release intracellular calcium stores leading to enhanced calmodulin kinase (CamK) activity, leading to synaptic plasticity. (Image source: Gali et al., 2014)

Similarly, NGF upregulation causes phosphorylation of TrkA receptors which eventually can lead to the downstream activation of Akt and ERK1/2 pathways. This in turn can rescue striatal neurons from undergoing apoptosis on VD administration (Romon et al., 2010) as shown in **Fig. 35**.

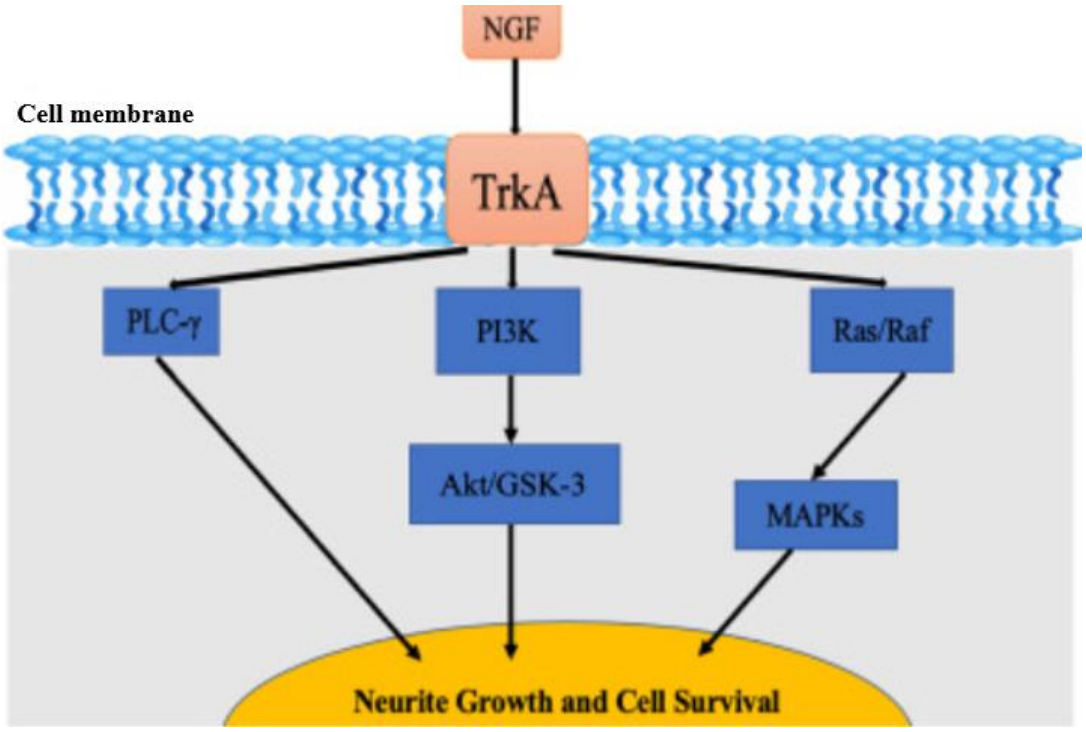

Fig.35. Interactions of NGF with TrkA lead to the activation of major intracellular signaling pathways. NGF binding to TrkA receptor leads to dimerization and autophosphorylation. The linker Shc binds to phospho-Y490 on Trk and to a Grb2-SOS complex. SOS is a nucleotide exchange factor that activates Ras by replacing GDP with GTP. Activated Ras interacts directly with the serine–threonine kinase Raf. The activated Raf leads to the sequential activation of MAPK kinase (MEK), the mitogen-activated protein kinase-ERK kinase (MAPK). MAPK translocates to the nucleus, where it phosphorylates transcription factors, promoting neuronal cell differentiation. Activation of phosphatidylinositol 3-kinase through Ras or Gab1 promotes survival and growth of neurons. Activation of phospholipase C- γ 1 (PLC- γ 1) results in activation of Ca 2+ - and protein kinase C-regulated pathways that promote synaptic plasticity. (Image source: Skaper, 2012)

Previous studies have proposed that VD upregulates the expression of BDNF and NGF in similar age-related neurological conditions like AD and PD (Gezen-Ak et al., 2014; Khairy and Attia, 2021). They have shown that the upregulation of neurotrophins takes place via VDR signaling (Alsulami et al., 2020; Cornet et al., 1998). In our study also, we have provided a similar effect of VD-mediated enhancement in VDR expression and a conjoint positive effect of VD-VDR signaling on neurotrophins gene expression. The findings of chapters 3 and 4 also investigated the VD anti-oxidant effect in the striatum and the cortex. VD decreased the expression of anti-oxidants like glutathione peroxidase

(GpX4) and catalase (Cat). These anti-oxidants are known regulators of oxidative stress as they eliminate excess number of oxygen-free radicles as shown in **Fig. 36.**

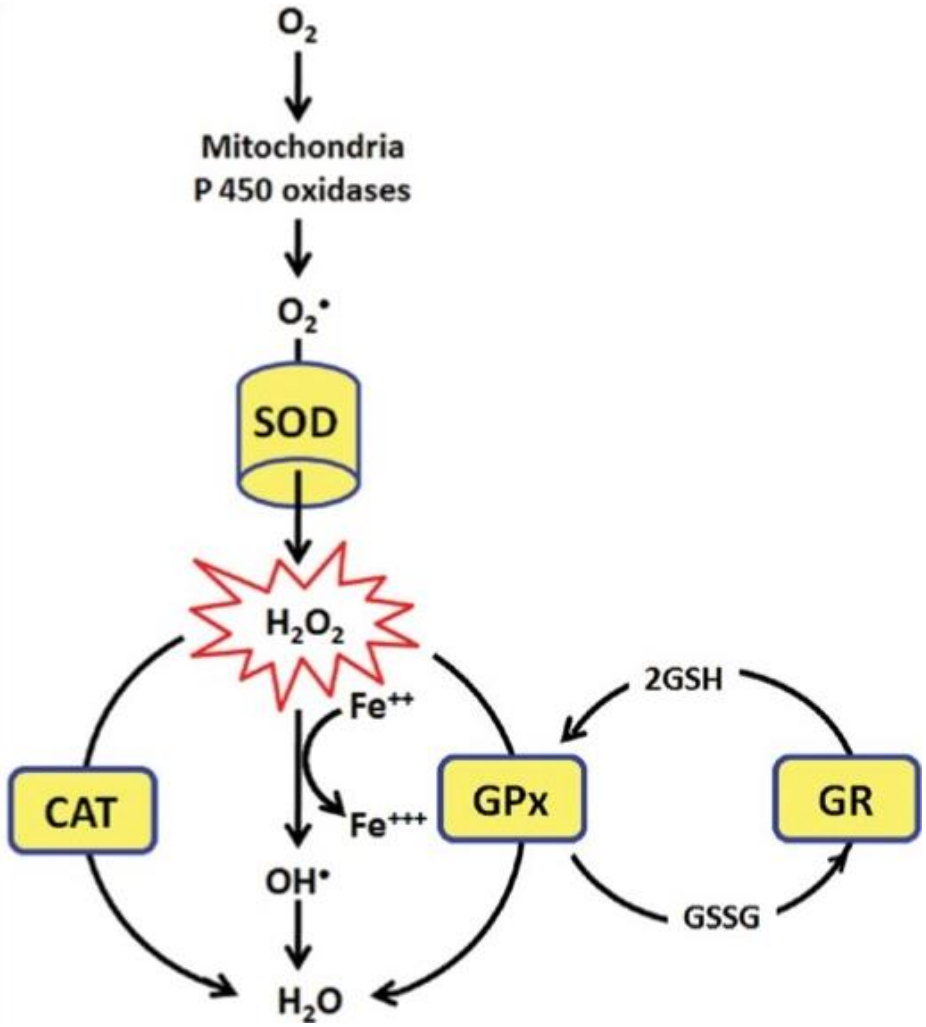

Fig.36. Schematic representation of anti-oxidants in protecting the cells from oxidative stress. Superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) are the main endogenous enzymatic defense systems of all aerobic cells. They give protection by directly scavenging superoxide radicals and hydrogen peroxide, converting them to less reactive species. SOD catalyzes the dismutation of superoxide radical ($\bullet O_2$) to hydrogen peroxide (H_2O_2). Although H_2O_2 is not a radical, it is rapidly converted by fenton reaction into •OH radical which is very reactive. GpX neutralizes hydrogen peroxide by taking hydrogens from two GSH molecules resulting in two H_2O and one GSSG. GR then regenerates GSH from GSSG. CAT the important part of enzymatic defense, neutralizes H_2O_2 into H_2O. (Image source: Pandey and Rizvi, 2010)

In chapter 4, we provided an anti-inflammatory function of VD in HD. VD administration in HD mice significantly decreased the gene expression of TNF-α and IL-6 in the cortex and in the striatum (**Fig. 32**). In our study, we also found a profound reduction in the gene expression of NF-κB. Since NF-κB is a critical mediator of pro-inflammatory gene induction, it is proposed that VD intervention in HD can combat cytokine storm as shown in **Fig. 37**.

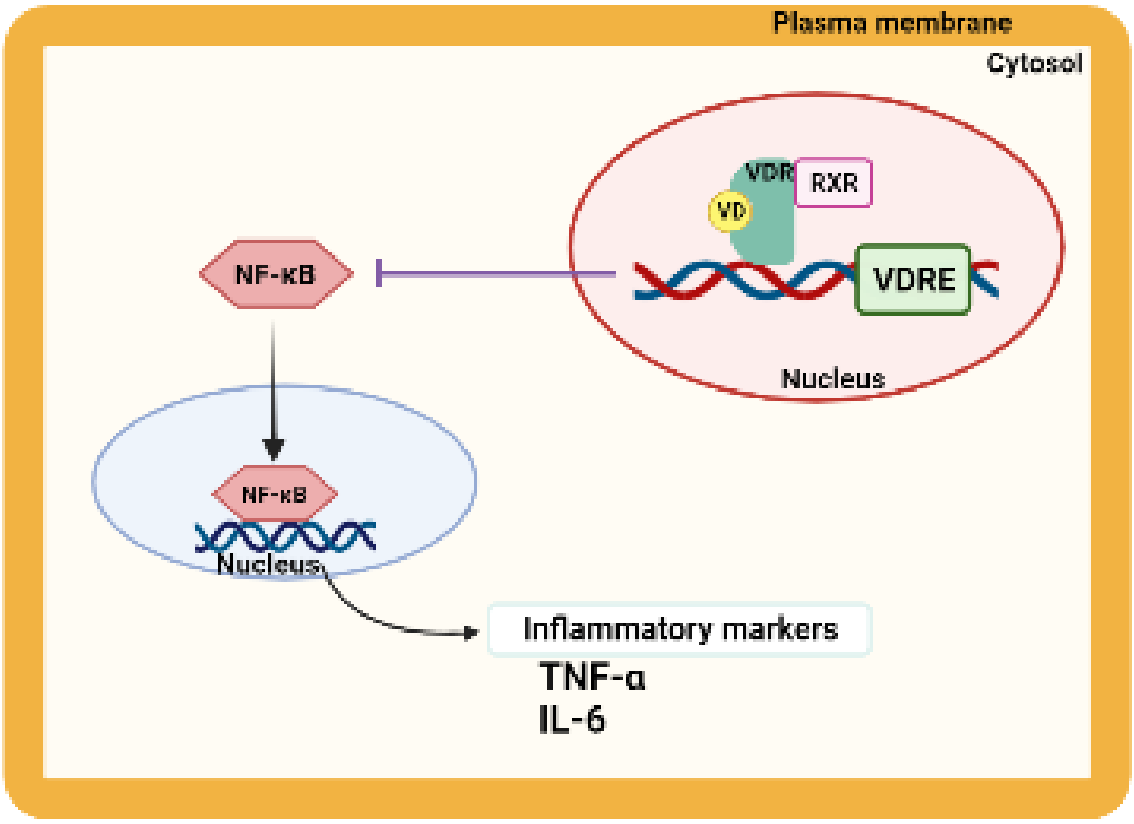

Fig. 37. Suppression of NF-κB pathways by VD supplementation. VDR: Vitamin D receptor; VDRE: Vitamin D response element; NF-κB: nuclear factor kappa B; D: $1,25(OH)_2$-Vitamin D3; IL-6: interleukin 6; TNF-α: tumor necrosis factor-α.

5.4. Cross-talk between VDR, TCR-β subunit and α7 nAChRs in HD:

Cholinergic dysfunction is a prominent feature of HD (Tata et al., 2014). In this dissertation we have shown that TCR-β is related with the function of α7 nicotinic acetylcholine receptor (α7 nAChRs) as we have shown that the expression of brain resident TCR-β expression gets upregulated in HD. From a previous study, it was evident that TCR activation downregulate the expression of α7 nAChRs from neocortex and prefrontal cortex

(Komal et al., 2014). Previous studies have demonstrated that activation of α7 nAChRs balances cognition and memory by modulating the kinases like Protein kinase A (PKA) and Src-family kinases like Lck and Fyn kinase (Komal et al., 2015, 2014; Komal and Nashmi, 2015). Taking this into consideration we have shown that VD intake by HD mice rescued the cholinergic activity with a concomitant decrease in the expression of the TCR-β subunit with a simultaneous decrease in the activity of acetylcholine esterase (AChE). Overall our study showed the anti-oxidant, anti-cholinergic, anti-inflammatory, and anti-apoptotic properties of VD in HD.

Chapter 6

Conclusion

The Asian prevalence of HD seems to be on high rise, though the increased prevalence of HD is mainly reported from Europe, Australia and North America. The present treatment for HD is tetrabenazine (TBZ), a drug that is believed to treat only the symptoms of HD (Frank, 2010; Grigoriadis et al., 2017). In this regard, multiple evidence pinpoints towards the therapeutic potential of VD as a mean of minimizing the neurotoxic conditions observed across myriad neurological disorders like Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) (Banerjee and Chatterjee, 2003; Chel et al., 2013; Moretti et al., 2018; Holick et al., 2011).

In recent years, several scientific studies have linked VD deficiency as one of the major risk factor involved with the onset of many age-related neurological disorders (Berridge, 2017). A high prevalence of VD deficiency is also observed in HD, a neurodegenerative disease characterized by a decrease in neurotrophins synthesis, increase in oxidative stress, inflammation, and apoptosis (Chel et al., 2013; Wilson et al., 2023). On the basis of the data reported in this thesis, VD can be considered an easily available and cheap neutraceutical that may delay the process of neurodegeneration in HD (Lima et al., 2018; Mohamed et al., 2015). Based on the findings of this thesis, the following key conclusions are drawn:

- 500IU/kg/day dosage of VD rescued behavior deficits observed in HD mice.
- VD administration to HD mice potentiated striatal gene expression of neurotrophins such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF).
- VD was effective in combating oxidative stress and neuroinflammation through the reduction of the gene expression of anti-oxidants and anti-inflammatory markers in cortical and striatal brain regions of HD mice.
- VD administration signigicantly reduced the gene expression of NF-κB in the striatum of HD mice.
- VD potentiated cholinergic signaling under neurotoxic conditions induced by 3-NP and caused reduction in the cholinesterase activity, increased the protein/gene expression of α7nAChRs with a concomitant decrease in the gene expression of T-cell receptor beta subunit expression in two important regions of the brain known for attention, memory and cognition namely, the striatum and cortex.

- ❖ The beneficial effects of VD occurred via an enhancement in the VDR signal transduction pathways.

6.1. **Limitations:**

The present study emphasized on the neuroprotective effects of VD in HD but has some limitations. The study has addressed the beneficial effect of only one dose in a murine model induced by 3-NP which mimics HD like symptoms. I have not undertaken a direct comparison on the dose dependent effect of VD on various behavior phenotypes. It is possible that the additional valuable factors of VD, not reported here, may be identified by utilizing a genetic model of HD like YAC128. Our study also lacks data in determining the advantages of VD supplementation in Indian HD patients. It will be helpful in translating our findings from murine model to human diseases that will showcast the differences between rodent and human biology, disease pathology and, VD metabolism. It will be necessary that such clinical research are carried out with appropriate controls as it is absolutely a requirement to validate the efficacy and safety of VD supplementation in Indian HD patients. The limitations of the present study also arose due to the complexity of VD-VDR interaction that activates many downstream signal transduction pathways.

Specific Contributions

- In the present thesis, I showed VD's neuroprotective function in HD that occurred via VDR mediated signal transduction pathway.
- It is the first report that determined the beneficial effect of VD on the behavior phenotypes induced by 3-nitropropionic acid (3-NP) model of HD.
- It is the first report which elucidated the positive effect of VD on the neurotrophins gene expression in HD.
- It is the first evidence that determined the anti-oxidant, anti-inflammatory, anti-cholinergic, and anti-apoptotic activity in HD.

Future directions

1. Our results indicate that VD regulates the activity of acetylcholinesterase (AChE) and the expression of α7 nicotinic acetylcholine receptors (α7 nAChRs) which in turn can enhance synaptic function. These results can be followed up by exploring whether VD supplementation can maintain synaptic plasticity in Huntington's disease (HD).
2. VD is known to mediate a cross-talk between genomic and non-genomic pathways. A comprehensive analysis of these signal pathway by which VD shows its beneficial effect in HD needs validation.
3. An exploration of the specific contribution of VD-mediated signaling needs to be tested using a genetic *in-vivo* and *in-vitro* model in HD.

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List of Publications

1. **Manjari SKV**, Sharon Mariam Abraham, Omalur Eshwari, Kishore Golla, Priya Jhelum, Shuvadeep Maity and Pragya Komal; Tremendous Fidelity of Vitamin D3 in Age-related Neurological Disorders (2024). *Molecular Neurobiology*. 10.1007/s12035-024-03989-w.

2. **SKV Manjari**, Sharon Mariam Abraham, Poornima R, Rajneesh Kumar Chaturvedi, Shuvadeep Maity and Pragya Komal; Unprecedented effect of Vitamin D3 on T-cell receptor beta subunit and alpha7 nicotinic acetylcholine receptor expression in 3-nitropropionic acid-induced mouse model of Huntington's disease (2023). *IBRO Neuroscience Reports.* Vol.15. Pg: 116-125. doi: https://doi.org/10.1016/j.ibneur.2023.07.001.

3. Pragya Komal, **Manjari SKV**, Raad Nashmi; (Invited Review); An opinion on the debatable function of brain resident immune protein, T-cell receptor beta subunit in the central nervous system (2022). *IBRO Neuroscience Reports.* Vol.13. Pg: 235-242. doi: https://doi.org/10.1016/j.ibneur.2022.09.003.

4. **Manjari SKV**, Shuvadeep Maity, Poornima R, Suk-Yu Yau, Vaishali K, David Stellwagen and Pragya Komal; Restorative action of Vitamin D3 on motor dysfunction through enhancement of neurotrophins and antioxidant expression in the striatum (2022). *Neuroscience.* Vol.492. Pg: 67-81. doi: https://doi.org/10.1016/j.neuroscience.2022.03.039.

5. Anurag Gautam, Pragya Komal, Ram Sevak Singh, Prabhat Gautam, **S. K. V. Manjari**, R. S. Ningthoujam; Hardcore producer of polyvinyl alcohol as reducer for the formation of gold nanoparticles (2021), *Journal of molecular Liquids.* Vol. 334. Pg: 16112. doi: https://doi.org/10.1016/j.molliq.2021.116112

List of Conference Proceedings

1. Attended the "Synergistic training program utilizing the scientific and technological infrastructure (STUTI)' workshop conducted by the Department of Science and Technology (DST) at the University of Hyderabad in January 2023.
2. Presented poster on the topic "Vitamin D3 administration alleviates native immune receptor, t-cell receptor beta subunit expression and potentiates cholinergic signaling in a mouse model of Huntington's disease" in the Indian Academy of Neuroscience conference (IAN) in December 2022.
3. Presented poster on the topic "Vitamin D3 administration alleviates native immune receptor, T-cell receptor beta subunit expression and potentiates cholinergic signaling in a mouse model of Huntington's disease" at Society for Neurochemistry India conference (SNCI) in November 2022; **facilitated with the best poster award.**
4. Presented a poster on the topic "Vitamin D3 Supplement Rescues Motor Disability in 3-Nitropropionic acid Induced Mouse Model of Huntington's Disease" in Laboratory Animals Scientists Association Conference–2022 in May 2022.
5. Presented an oral presentation on the topic "Vitamin D supplementation attenuates oxidative stress, motor impairment, neuromuscular coordination, and spatial memory in a 3-nitropropionic acid-induced mouse model of Huntington's disease" in the Society of Neurochemistry India 35th Annual Conference in December 2021.
6. Manjari SKV, and Pragya Komal* (Corresponding author); 7th Asian Oceanian Congress on Clinical Neurophysiology (AOCCN); poster; Prophylactic effect of Vitamin D3 supplementation in 3-nitropropionic acid-induced mouse model of Huntington's disease. Abstract published as conference proceedings;"2021.
7. Presented a poster on the topic "Therapeutic activity of Vitamin D3 supplementation on oxidative stress, motor impairment, neuromuscular coordination and spatial memory in 3-nitropropionic acid-induced mouse model of Huntington's disease" in "Virtual International Conference on Novel and Alternative Therapeutics for Neurodegenerative Diseases Mediated Through Unfolded protein response (UPR)" (IBRO) in September 2021; **facilitated with the best poster award**.
8. Presented a poster on the topic "Unprecedented expression of brain resident T-cell receptor beta and cholinergic alpha 7 nicotinic acetylcholine receptor in a

neurodegenerative disorder" at 4th Macquarie Neurodegeneration meeting, Australia, in September – 2021.

9. Presented a poster on the topic "Vitamin D3 attenuates oxidative stress, motor dysfunction, neuromuscular coordination, and spatial memory in a mouse model of Huntington's disease" in the Canadian Association of neurosciences (CAN-CAN) in August 2021.
10. Presented poster on the topic "Therapeutic effects of Vitamin D supplementation in a mouse model of Huntington's disease" at the Society of Neurochemistry India's 34 th annual conference in December 2020.
11. Presented a poster on the topic "Vitamin D intake enhances Vitamin D receptor expression in the striatum and rescues memory and motor dysfunction in a mouse model of Huntington's disease" at the IAN international e-conference in October 2020.
12. Presented a poster on the topic "Vitamin D intake enhances Vitamin D receptor expression in the striatum and rescues memory and motor dysfunction in a mouse model of Huntington's disease" in 3rd Macquarie Neurodegeneration meeting in October – 2020.
13. Presented a poster on the topic "Vitamin D3 supplement rescues motor disability in a 3-nitropropionic acid-induced mouse model of Huntington's disease" in the Indian Academy of Neuroscience (IAN) – 2019 at AIIMS – New Delhi in 2019; **facilitated with the best poster award**.

Biography of the Candidate

Ms. S K Venkata Manjari is a full-time Ph.D. student in Department of Biological Sciences, BITS-Pilani, Hyderabad Campus, under the supervision of Dr. Pragya Komal. She obtained her B. Tech (Bio-Technology) from Andhra University, Vishakhapatnam, India and qualified GATE-2015 securing an All-india Rank of 622. Then, she did M. Tech (Bio-Technology) from Birla Institute of Technology, Mesra, India. With her diverse knowledge in the field of life sciences and biological methods, she took admission in Ph.D. in the department. During her training as a research scholar, she also practiced teaching assistance wherein she handled laboratory courses for B.E. and M.E. Biotechnology students at BITS-Pilani, Hyderabad. She is well versed in various microbiology, molecular biology, animal handling and analytical techniques. During her tenure she is a recipient of CSIR-SRF (2021-2024). She has presented her work in several national and international conferences. She recived best poster awards in different conferences for the work she has presented. Currently, her research interests are focused on therapeutic activity of different drugs by targeting various signalling pathways involved in various neurological disorders.

Biography of the Supervisor

Dr. Pragya is presently working as an assistant professor in the Department of Biological Sciences, BITS-Pilani, Hyderabad. During her Ph.D. and post-doctoral training in the field of neuroscience and mental health at the University of Victoria (British Columbia), McGill University (Montreal) and at the hospital of sick children (Toronto), she discovered the molecular underpinning mechanism of ionotropic receptor regulation in healthy and diseased brains. She undertook the functional analysis of ligand-gated ion channels like cholinergic and glutamate receptors where she measured synaptic transmission and synaptic plasticity via whole-cell patch-clamp recording. She has vast experience in cell biology, electrophysiology, and neurobiology of mental disorders like schizophrenia and Huntington's disease (HD). She has published her research in several international journals including the Journal of Neuroscience, Journal of Physiology, and Neuroscience. She has also successfully established an optogenetics-enabled electrophysiology facility under the DBT-builder interdisciplinary project at the BITS-Hyderabad campus. As a young investigator, she is the recipient of an international travel award, an early career award, and a Young Maternity Parenthood grant award from the International Brain Research Organization (IBRO). Her lab presently focusses on the therapeutic potential of Vitamin D3 in neurologicl disorders like Huntington's disease and schizophrenia. Her research is funded by national and international funding agencies like DST, DBT and IBRO.

NEUROSCIENCE
RESEARCH ARTICLE
S. K. V. Manjari et al. / Neuroscience 492 (2022) 67–81

Restorative Action of Vitamin D3 on Motor Dysfunction Through Enhancement of Neurotrophins and Antioxidant Expression in the Striatum

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Abstract—A number of studies has explored a positive correlation between low levels of serum Vitamin D_3 (VD; cholecalciferol) and development of neurodegenerative diseases including Huntington's disease (HD). In the present study, the prophylactic effect of VD on motor dysfunction was studied in an experimental model of HD. An HD-like syndrome was induced in male C57BL/6 mice through an intraperitoneal injection (i.p) of 3-NP for 3 consecutive doses at 12 h interval of time as described previously (Amende et al. 2005). This study investigated the -*in-vivo* therapeutic potential of VD (500 IU/kg/day) supplementation on movement, motor coordination, motor activity and biochemical changes in this HD model. Mice were divided into four groups: Group I: Control (saline); Group II: 3-NP induced HD (HD); Group III: Vitamin D_3 (VD) and Group IV: 3-NP induced + post Vitamin D_3 injection (HD + VD). All groups of mice were tested for locomotion, gait analysis and rotarod performances over a span of 30-days. VD administration rescued locomotor dysfunction and neuromuscular impairment in HD mice with no change in gait dynamics. In addition, administration of VD to 3-NP treated mice led to a significant enhancement in the expression of key neurotrophic factors including brain-derived neurotrophic factor (BDNF) and nerve-growth factor (NGF), the Vitamin D receptor (VDR), and antioxidant markers (catalases [Cat] and glutathione peroxidase [GpX4]) in the striatum, suggesting a detoxification effect of VD. Altogether, our results show that VD supplementation induces survival signals, diminishes oxidative stress, and reduces movement and motor dysfunction in HD.

Key words: Huntington's disease (HD), vitamin D_3 (VD), 3-nitropropionic acid (3-NP), cholecalciferol, neurotrophic factors, antioxidants.

INTRODUCTION

Huntington's disease (HD) is a progressive neurodegenerative disorder with a prevalence in the range of 1/10,000–1/20,000 in the Caucasian population and 0.4/1,00,000 in Asian populations respectively (Pringsheim et al., 2012; Chel et al., 2013; Baig et al., 2016; Rawlins et al., 2016). Extensive efforts have been made to understand the molecular, cellular, and system-level changes which occur during the progression of disease and their contribution towards striatal atrophy. The selective loss of medium spiny neurons (MSN) is known to be the main causes for motor disorders associated with Huntington's disease (HD) (Gil and Rego, 2008; Gil-Mohapel, 2012; Lewitus et al., 2014). The loss of γ-amino butyric acid (GABA) signaling from the MSNs causes circuit dysfunction, which results in involuntary movements, postural instability, lack of coordination,

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Abbreviations: 25(OH)VD_3, Calcidiol; 3-NP, 3-nitropropionic acid; AD, Alzheimer's disease; BDNF, Brain-derived neurotrophic factor; Cat, Catalases; cDNA, Complementary DNA; GpX4, Glutathione peroxidases; HD, Huntington's disease; HRP, Horseradish peroxidase; *Htt*, Huntingtin gene; i.p, Intraperitoneal; mHTT, mutant Huntingtin protein; MSN, Medium spiny neurons; NGF, Nerve-growth factor; PD, Parkinson's disease; ROS, Reactive oxygen species; RT-PCR, Real-Time polymerase chain reaction; SOD1, Superoxide dismutase 1; SOD2, Superoxide dismutase 2; VD, Vitamin D_3; VDR, Vitamin D receptor; ANOVA, Analysis of variance; SEM, Standard error of the mean; GSH, Glutathione; 1-α-25-$(OH)_2$-VD_3, Calcitriol.

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and cognitive and psychiatric impairments (Gil and Rego, 2008). HD is a monogenic, autosomal dominant disorder caused by expansion of a trinucleotide CAG sequence (encoding glutamine) in the first exon of the huntingtin (*Htt*) gene, located on chromosome 4, with an inverse correlation between repeat length and age of onset of symptoms (Gil and Rego, 2008; Gil-Mohapel, 2012; Blumenstock and Dudanova, 2020). The polyglutamine expansion in huntingtin protein (HTT) causes mitochondrial dysfunction, neuro-inflammation and oxidative stress which ultimately lead to the death of striatal neurons (Brouillet et al., 2005; Gil and Rego, 2008; Blumenstock and Dudanova, 2020). Mutant huntingtin protein aggregates in the striatum impairs cellular processes like mitochondrial function, initiates autophagy and proteostasis, and ultimately enhances oxidative stress in HD (Maity et al., 2022). Striatal damage is also known to be induced by the mitochondrial toxin, 3-nitropropionic acid (3-NP) which reproduces symptoms of HD in animals, including hypokinetic motor impairment which mimic some of the neuropathophysiological symptoms of HD (Beal et al., 1993; Brouillet et al., 2005; Kumar, Kalonia, and Kumar, 2009; Túnez et al., 2010; Brouillet, 2014). 3-NP is an irreversible inhibitor of mitochondrial succinate dehydrogenase in the tricarboxylic acid cycle (TCA) (Brouillet et al., 2005; Duran-Vilaregut et al., 2009; Túnez et al., 2010). The potential utility of the 3-NP model of striatal degeneration comes from studies that show mitochondrial impairment, energy depletion, and oxidative stress are the key players in HD pathogenesis (Beal et al., 1993; Vis et al., 1999; Kumar, Kalonia, and Kumar, 2009; Johri and Beal, 2012). The high energy demand by neurons of the central nervous system makes them most vulnerable to the metabolic alterations observed in HD patients (Johri and Beal, 2012; Paul and Snyder, 2019).

In the last decade, a potential link has been explored between Vitamin D_3 (VD or cholecalciferol) deficiency and neurodegenerative disorders (Holick et al., 2011; Chel et al., 2013; Molnár et al., 2016; Koduah et al., 2017; Amrein et al., 2020). Vitamin D_3 (VD) is a neurosteroid hormone that shows neuroprotection effects in animal and cell-culture models of Parkinson's and Alzheimer's disease (Kim et al., 2006; Sanchez et al., 2009; Nimitphong and Holick, 2011; Mohamed et al., 2015; Calvello et al., 2017; Koduah et al., 2017; Lima et al., 2018; AlJohri et al., 2019; Bivona et al., 2019; Rodrigues et al., 2019). Calcitriol, which is the active form of VD, exerts its neuroprotective role via Vitamin D receptor (VDR) (Taniura et al., 2006; Butler et al., 2011; Bankole et al., 2015; Ricca et al., 2018).

Evidence suggests that VD supplementation increases the release of neurotrophic factors like nerve growth factor (NGF) and brain derived neurotrophic factor (BDNF) in neurodegenerative diseases like Parkinson's and Alzheimer's disease to reduce neuronal death by apoptosis or necrosis (Kim et al., 2006; Allen et al., 2013; Baydyuk and Xu, 2014; Mohamed et al., 2015). These neurotrophins also promote synaptic function and survival of several neuronal populations, including striatal neurons that are the primary affected cells in HD (Zuccato et al., 2001; Zuccato and Cattaneo 2007). Oxidative stress markers are also observed as a hallmark of neurodegenerative disorders like HD (Johri and Beal, 2012; Brouillet 2014; Lima et al., 2018; Paul and Snyder 2019). This can be identified by the effect of oxidative stress on certain antioxidants like superoxide dismutase (SOD), glutathione peroxidase (GpX), and catalase (Cat). Studies suggest that VD supplementation has a regulatory effect on oxidative stress which leads to the survival of neurons (Lima et al., 2018; Bakhtiari-Dovvombaygi et al., 2021; Latham et al., 2021). Though VD supplementation is readily available and affordable, little is known about its potential beneficial effects in HD. Limited evidence is available to correlate VD deficiency with HD and whether high Vitamin D supplementation affects motor function in HD has not been established (Chel et al., 2013). The cellular mechanism responsible for neuroprotection of Vitamin D supplementation also remains uncertain. Therefore, the present study was undertaken with the aim to explore the effect of 500 IU/kg of Vitamin D supplementation on motor dysfunction following administration of 3-nitropropionic acid (3-NP).

EXPERIMENTAL PROCEDURES

Animal procurement

Ten to twelve weeks old male C57BL/6 mice (average weight; 26 ± 3 g) were acquired from Sainath Agencies, Hyderabad, India. Animals were group housed (2 mice per cage) with *ad libitum* access to food and water. They were kept in a 12 h light/12 h dark cycle at 25 ± 2 °C. All the animal experiments were carried out with the approval of the Institutional Animal Ethics Committee (IAEC), BITS – Pilani, Hyderabad, India. All efforts were made to minimize the number of animals used and their suffering.

Study design

All the animals were acclimatized for 5 days and then received behavioral training for 7 days prior to treatment. Animals were then randomly divided into four experimental groups (Group I to Group IV; Table 1) and given injections of 3-nitropropionic acid (3-NP) and/or Vitamin D_3 (VD or cholecalciferol) (Fig. 1). 3-NP was given by three intraperitoneal injections (i.p) of 25 mg/kg, every 12 h, for a cumulative dose of 75 mg/kg as described previously with minimal modification (Fernagut et al., 2002; Amende et al., 2005). VD was given i.p. daily for 15 days at 500 IU/kg/day.

Table 1. The four different experimental groups of C57BL/6 male mice (3–4 months old)

Animal	Groups
	Control (1× saline) (**Group I**)
	3-NP (75 mg/kg) (**Group II**)
	Vitamin D (500 IU/kg) (**Group III**)
	3-NP (75 mg/kg) + Vitamin D (500 IU/kg) (**Group IV**)

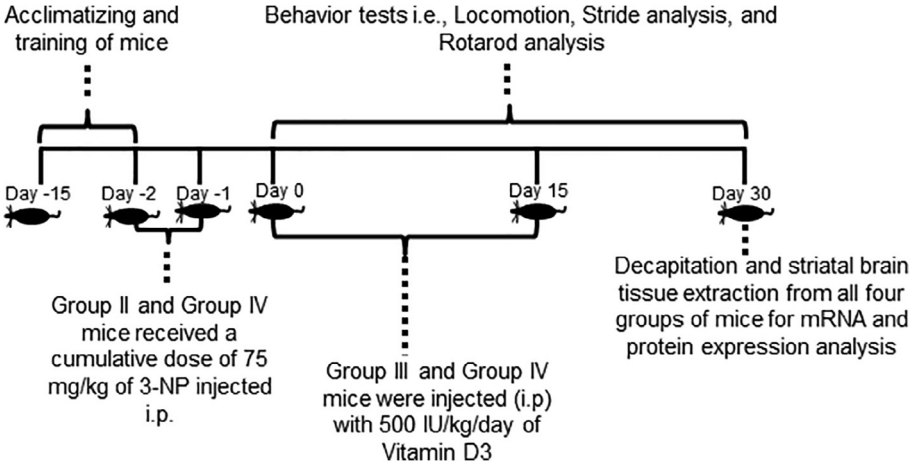

Fig. 1. Timeline and design for the behavioral study. C57BL/6 male mice (3–4 months) were trained for 7 days in the behavioral tasks and thereafter injected (i.p) with 3-nitropropionic acid (3-NP) in 3 doses of 25 mg/kg at 12 h intervals (cumulative dose of 75 mg/kg; Group II and IV). VD (500 IU/kg/day) supplementation was given to Group III (only VD) and Group IV mice after post-injection with 3-NP (HD + VD -) for 15 days (Day 1–Day 15). Behaviors analysis was conducted from Day 1 to Day 30. On the 30th day, mice were sacrificed and the striatal brain tissues were extracted for gene and protein expression analysis.

Experimental design

The mice were randomly divided into four experimental groups for behavior and biochemical assay. (Table 1)

i. **Group I:** Control group mice (C57BL/6) injected with saline.
ii. **Group II:** 3-NP induced mice by i.p. injection (3-NP; 75 mg/kg) without VD-treatment (HD).
iii. **Group III:** Mice injected solely with 500 IU/kg/day Vitamin D_3 (VD) for 15 days.
iv. **Group IV:** Post-intraperitoneal injection of 500 IU/kg/day of VD to 3-NP (75 mg/kg) pre-injected mice for 15 days (HD + VD).

Drugs and reagents

i) **Cholecalciferol (Vitamin D_3; VD)** was purchased from Sigma-Aldrich, India (Cat No: C9756) and dissolved in 1% ethanol (diluted with sterile saline) on the day of injection (Mohamed et al., 2015). Mice were administered with 500 IU/kg (12.5 μg/kg/day) i.p. of VD as reported previously (Chabas et al., 2013; Gueye et al., 2015; Kolla and Majagi, 2019). Briefly, VD was administered to the Group III (only VD) mice and to Group IV (HD + VD) mice. Group IV mice (HD + VD) were given 24 h recovery time from previous 3-NP induction. Then the VD injections were carried out 24 h after the last dose of 3-NP daily for 15 days to Group IV mice (from 0 to 15th day, Fig. 1 and Table 1).

ii) **3-nitropropionic acid (3-NP)** was purchased from Sigma-Aldrich, India (Cat No.: N22908). Stock solutions of 3-NP (3 mg/ml) were prepared in 0.1 M phosphate buffered saline solution and were injected intraperitoneally at 25 mg/kg (3-NP; cumulative dose of 75 mg/kg) thrice at 12 h intervals to respective groups of mice as described previously (Fig. 1 and Table 1). Controls were treated with three doses of saline at 12 h intervals. In this study, we used a subacute dose of 3-nitropropionic acid dose as reported previously by Amenda et al., 2005 with minimal modification. This protocol is based on previous published studies who used 50 mg/kg i.p. injection of 3-NP for 5 days (Kim and Chan, 2001; Fernagut et al., 2002). To model a subacute exposure to 3-NP, a cumulative dose of 75 mg/kg dose of 3-NP was undertaken (Kim and Chan, 2001; Fernagut et al., 2002).

Behavioral evaluations

A total of 80 mice were used for behavioral experiments. Mice were initially assessed for locomotion and gait as previously reported by Amende et al. (2005) and Fernagut et al. (2002). A separate cohort was used to evaluate the effects on locomotion and rotarod performance. Only two behavioral tests were done on a given set of animals. Protocols for behavioral tests were:

i) Assessment of locomotor activity

The locomotor activity was monitored using an actophotometer as described previously (Digital Photoactometer cage; Dolphin, 2009), using the number of beam breaks as the measure of movement for each animal (Kumar et al., 2009). Locomotion was measured over a 5 min period, and baseline readings were taken before the respective drug injections (Fig. 2).

ii) Estimation of gait by stride length analysis

Stride length analysis was done to determine the choreatic movement in mice by marking the animals' forepaws and hind paws with ink (red for forelimbs and blue for hind limbs; Fig. 3). The animals were allowed to move on a strip of paper (4 cm wide and 56 cm long) placed on a brightly lit runway leading to a darkened box. Stride length was measured manually as the distance between two paw prints as described previously (Fernagut et al., 2002). Forelimb stride length measurement was first measured for all mice followed by hind limb stride length on a new strip of paper.

iii) Assessment of motor coordination by rotarod analysis

The integrity of motor coordination was measured using the rotarod as described previously (Kumar et al., 2009). Briefly, the rotarod apparatus consists of a long rotating rod of 90 cm long and 3 cm in diameter. The apparatus was divided into three different compartments

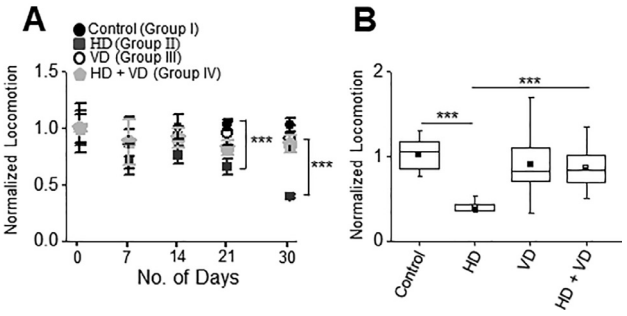

Fig. 2. VD supplementation rescues locomotor performance in HD mice. **(A)** Vitamin D supplementation (VD; cholecalciferol; 500 IU/kg/day) significantly reversed the loss of locomotor activity due to 3-NP treatment in HD mice (HD + VD vs HD; $n = 8–10$; $p < 0.001$, two-way repeated measures ANOVA). All data are normalized values against the initial day for each group and is represented as mean ± SEM. **(B)** On 30th day, a significant decrease in locomotion activity in HD mice (Group II) was observed as compared to Control mice (Group I), which was reversed significantly upon VD supplementation (HD vs Control; $n = 10$, $p < 0.001$, Tukey's *post-hoc* analysis). Data is represented as box-and-whisker plots depicting median with first and third quartiles; shaded square is the mean for each group and whiskers represents 5th and 95th percentile values.

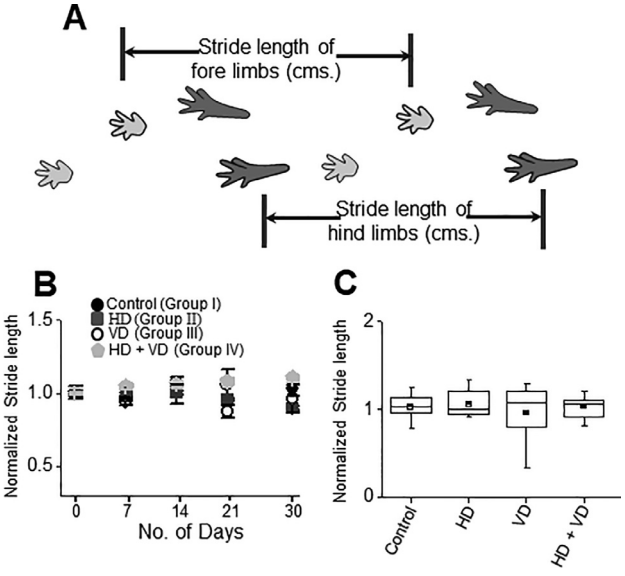

Fig. 3. 3-NP or VD administration have no effect in gait dynamics. **(A)** Schematic representation of paw prints, with gait assessed by stride length analysis. Left and right paws of individual mice were coated with non-toxic ink and mice were allowed to walk on a sheet of oriental white paper. Overall stride of the mice is represented as the average stride of forelimbs and hind limbs. Stride length was determined as the distance between two consecutive paw prints. **(B)** 3-NP (i.p; 75 mg/kg) induction produced no change in forelimb and hind limb performance in HD mice (Group II) as compared with Group I (Control) mice across a span of 30 days ($n = 4–10$, $p = 0.4$, two-way ANOVA). VD supplementation also showed no significant effect on gait dynamics in 3-NP induced HD mice ($n = 4–10$, $p = 0.4$, two-way ANOVA). All data are normalized value against initial day for each group and is represented as mean ± SEM. **(C)** On 30th day, no effect of VD was observed on the stride length performance of 3-NP pre-treated mice (Group IV; HD + VD) as compared to HD (Group II) mice ($n = 10$, $p = 0.70$, Tukey's *post-hoc* analysis). Data is represented as box-and-whisker plots depicting median with first and third quartiles; shaded square is the mean for each group and whiskers represents 5th and 95th percentile values.

by a glass partition (Rota rod 3 compartments, Dolphin, 2019). The rod rotation speed was set initially at 35 rotations per minute (RPM). Mice received training on the accelerating rod prior to treatment. After achieving criterion (no falls from the rotarod within 180 sec, mice were injected with either saline (Group I; Control), 3-NP (Group II; HD) or VD (Group III) or both (Group IV; HD + VD). After the respective injections, the treated mice were re-tested for 180 sec and the latency to fall was recorded and analyzed.

RNA isolation and cDNA preparation

On the 30th day, mice from respective groups were anesthetized using isoflurane (Rx, NoB506) and immediately decapitated for the extraction of striatal brain samples. Brain tissue was placed into 1 ml of RNAiso PLUS (Takara Bio) and sonicated on ice. 200 μl of chloroform was added and samples were centrifuged for 30 min at 12,000*g* at 4 °C (Eppendorf Refrigerated centrifuge, 542R). After isolation of the aqueous phase, an equal volume of isopropanol (Hi-Media Laboratories, Molecular biology grade, India) was added, incubated overnight at −20 °C and again centrifuged at 12,000*g* for 30 min at 4 °C. Samples were washed with 70% ice-cold ethanol and the obtained pellet was resuspended in nuclease-free water. DNase I (EN052, Thermo Scientific™, USA) treatment was performed to remove any DNA contamination. DNase-treated samples were made up to 400 μl using nuclease-free water. It was followed by sample purification using 1/10th volume of 3 M sodium acetate and 2× volume of phenol: chloroform: isoamyl alcohol (Sisco Research Laboratories Pvt. Ltd., India) and centrifuged for 2 min at maximum speed at 4 °C. The aqueous phase was isolated with addition of an equal volume of ice-cold 100% ethanol, followed by overnight incubation at −20 °C. The samples were again centrifuged at maximum speed for 15 min at 4 °C, then washed with 70% ice-cold ethanol and the obtained pellet was resuspended in nuclease-free water. The total concentration of purified RNA was estimated by the Nanodrop spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA). An equal amount of RNA from each group was used to reverse transcribe complementary DNA (cDNA) with the help of the Verso cDNA synthesis kit (Cat No: AB1453A, Thermo Scientific™, USA) as per manufacturer's instruction. Briefly, 500 ng of purified RNA was taken from each group for cDNA synthesis with the following reaction conditions: 42 °C for 1 h followed by 95 °C for 2 min. The obtained cDNA was used for semiquantitative PCR and real-time PCR (RT-PCR) (Fig. 5).

Analysis of mRNA expression for nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), and antioxidant marker genes

The sequences of neurotrophic genes (NGF and BDNF) of the mouse genome were obtained from NCBI. The sequences were deposited in the IDT primer quest tool to get the most suitable primer for gene analysis. For

antioxidant marker genes, we analyzed superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), catalase (Cat) and glutathione peroxidase 4 (GpX4). All the genes, primer sequences and amplicon sizes are listed in Table 2. Semiquantitative-PCR was performed using respective cDNA with gene specific primers to estimate the relative quantification of target genes. We used the following PCR condition to amplify NGF using 2X PCR master mix (Takara Bio) and 0.5 μM of each primer: 95 °C for 2 min; 35 cycles of 95 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s; and a final step of extension of 72 °C for 5 min. For antioxidant markers the PCR condition: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 60 °C for 45 s, 72 °C for 45 s; and a final step of extension at 72 °C for 10 min for SOD1, SOD2, and GpX4 whereas Cat amplification was carried out at 56 °C for 45 s. The PCR products were checked by electrophoresis on 1.5% agarose gel, visualized and quantified using Image software by keeping 18 s rRNA as a Control (housekeeping gene).

$$\textit{Relative Quantification of a gene} = \frac{\textit{Quantity of the required gene}}{\textit{Quantity of Housekeeping gene}}$$

Quantitative expression analysis for BDNF by Real-Time PCR (RT-PCR)

The expression of BDNF among the four groups of mice was assessed by RT-PCR in a CFX96 Touch Real-time PCR system (BioRad) using the GoTaq qPCR SYBR master mix (Cat No #A6001, Promega Corporation). The reaction mixture was prepared according to the manufacturer's protocol using ~12 ng of the cDNA template. Relative gene expression was quantified using the ΔCT method with respective primers (*BDNF* forward 5′-TCCTAGAGAAAGTCCCGGTATC-3′; reverse 5′-GCA GCCTTCCTTGGTGTAA-3′) and normalized to *18s* (forward 5′-ACGGAAGGGCACCACCAGGA-3′; reverse 5′-CACCACCACCCACGGAATCG-3′). We used the ΔΔCT method to determine the fold changes in the expression of BDNF (Livak and Schmittgen, 2001). Briefly, the threshold cycle (Ct) was extracted using Bio-Rad CFX Manager 3.1 software and relative gene expression was calculated as follows: fold change = $2^{-\Delta\Delta Ct}$, where ΔCt (cycle difference) = Ct (target gene) – Ct (Control gene) and ΔΔCt = ΔCt (treated condition) – ΔCt (Control condition) (Livak and Schmittgen, 2001).

Protein expression analysis for Vitamin D_3 receptor by western blot

On the 30th day, striatal brain tissue was extracted from all four groups of mice. The tissue was homogenized in the lysis buffer (150 mM sodium chloride, 1.0% TritonX-100, 0.5% sodium dodecyl sulfate and 50 mM Tris, pH 8.0). The protein concentration was determined using a Bradford protein assay kit (Bio-Rad, USA). We loaded equal amounts of protein (25 μg) run in a 12% gel, and then transferred to PVDF (Pall Corporation) membrane through a trans blot wet transfer system (Bio-Rad). The membrane was blocked using 5% BSA and incubated with respective primary and secondary antibodies for β-Actin Rabbit mAb (1:3000, CST#4970, Cell Signaling Technology); Vitamin D_3 Receptor Rabbit mAb (1:1500, CST#12550, Cell Signaling Technology); Anti-rabbit IgG-HRP-linked antibody (1:5000, CST#7074, Cell Signaling Technology). β-Actin served as a loading control. The signal intensities of the bands were captured using the fusion pulse gel documentation system (Eppendorf, USA). ImageJ software was used to quantify the band intensities.

Statistical analysis

Experimental data is represented as normalized values w.r.t to zero day for the respective groups of mice. Data in the figures are represented as box and whisker plots depicting the median with interquartile range; (central line: median; 25th and 75th quartiles; box: central shaded square: mean; whiskers: 5th–95th percentile values) to illustrate the distribution of normalized values for each

Table 2. Sequence of Primers used in PCR studies

Gene	Orientation	Sequence of primers (5′–3′)	Amplicon size
18s	Forward Reverse	ACGGAAGGGCACCACCAGGA CACCACCACCCACGGAATCG	127
NGF	Forward Reverse	GGCAGAACCGTACACAGATAG TGTGTCAAGGGAATGCTGAA	88
BDNF	Forward Reverse	TCCTAGAGAAAGTCCCGGTATC GCAGCCTTCCTTGGTGTAA	94
SOD1	Forward Reverse	CAGAAGGCAAGCGGTGAAC CAGCCTTGTGTATTGTCCCCATA	107
SOD2	Forward Reverse	TCCTAGAGAAAGTCCCGGTATC GCAGCCTTCCTTGGTGTAA	112
GPx4	Forward Reverse	GCCCAATACCACAACAGTAGA CCTGAACCACAGCGATGAA	108
Cat	Forward Reverse	AATTGCCTCCACACCTTCAC TCACCAAGCTGCTCATCAAC	107

respective group of mice (Group I to Group IV). Group data in the text and in the Supplementary tables are presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted using two-way repeated measures ANOVA, two-way ANOVA and one-way repeated measures ANOVA followed by either post hoc multiple pairwise analysis using Tukey's HSD tests or paired sample *t*-test. For non-parametric measurements, a Kruskal–Wallis test followed by an unpaired sample *t*-test was performed. $p < 0.05$ was set as threshold of significance ($^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$). All the data is displayed using Origin 8.1.

RESULTS

VD supplementation improves locomotor activity in a mouse model of HD

The impact of chronic supplementation of 500 IU/kg/day of VD on the locomotor activity in HD model mice was tested over a period of 30 consecutive days. An actophotometer was used to determine the total number of beam crossings for the evaluation of bradykinesia (Fig. 2) (Kumar et al., 2009). A repeated measures two-way ANOVA with power analysis showed a significant day effect ($F_{(3)} = 9.12$, $p < 0.001$, Fig. 2) and significant interaction between the groups and days ($F_{(3)} = 9.12$, $p < 0.001$, power value = 0.8). On the 7th day, there were no differences in the movement among the four experimental groups of mice. However, a consistent decrease of roughly 30% in the locomotory activity was observed on the 14th and 21st days in 3-NP injected HD mice (Group II) as compared to Control (Group I) mice; (14th day, 0.76 ± 0.08 vs 1.01 ± 0.11; 21st day 0.66 ± 0.07 vs 1.03 ± 0.05, $n = 8–10$, $p < 0.001$, Tukey's *post-hoc* analysis). On the 30th day it further deteriorated to 40% of Control values (Group II vs Group I; 0.40 ± 0.02 vs 1.03 ± 0.06, $n = 10$, $p < 0.001$, Tukey's *post-hoc* analysis). However, on the 14th and 21st days, Group IV mice supplemented with VD and pre-injected with 75 mg/kg of 3-NP showed a rescue in the locomotor activity near control levels and significantly above Group II mice (3-NP treated HD mice) (Group IV vs Group II; 14th day, 0.92 ± 0.09 vs 0.76 ± 0.08; 21st day, 0.83 ± 0.07 vs 0.66 ± 0.07, $n = 10$, $p < 0.001$, Tukey's *post-hoc* analysis). On the 30th day, Group IV mice on VD supplementation showed a robust enhancement by 1.2-fold in the locomotion performance as compared to HD mice (0.86 ± 0.07 vs 0.40 ± 0.02, $n = 10$, $p < 0.001$, Tukey's *post-hoc* analysis). To check the possibility that VD supplementation alone showed any improvement on the movement of the animals, VD injections solely were carried out in a Control group of mice (Group III). Interestingly, we found no significant difference between locomotion activity of VD administered mice as compared to Control mice for the entire timeline of 30 days (Group III vs Group I; 7th day, 0.87 ± 0.23 vs 0.87 ± 0.12; 14th day, 0.93 ± 0.09 vs 1.01 ± 0.11; 21st day 0.96 ± 0.1 vs 1.03 ± 0.05; 30th day, 0.91 ± 0.12 vs 1.03 ± 0.06, $n = 8–10$, $p = 0.7$, Tukey's *post-hoc* analysis, Fig. 2A, B). Further, no significant change in locomotion was observed between Group III (VD) and Group IV (HD + VD) mice nullifying the possibility of any chronic toxic side effect by 500 IU/kg/day of VD in Group III mice (Group III vs Group IV; 7th day, 0.87 ± 0.23 vs 0.88 ± 0.20; 14th day, 0.93 ± 0.09 vs 0.92 ± 0.09; 21st day 0.96 ± 0.10 vs 0.83 ± 0.07; 30th day, 0.91 ± 0.12 vs 0.86 ± 0.07, $n = 10$, $p = 0.9$, Tukey's *post-hoc* analysis, Fig. 2A, B). These results suggest the therapeutic potential of VD supplementation in rescuing locomotor dysfunction in HD mice. VD mediated a beneficial effect on movement occurred only when striatal neurons were subjected to neurodegeneration on 3-NP induction. Our results validate the *in-vivo* findings of Gueye et al. (2015) where a similar dose of Vitamin D_3 (VD, 500 IU/kg/day) resulted in a dramatic recovery in locomotor performance of animals subjected to spinal cord injury (Gueye et al., 2015).

Gait was unaltered in 3-NP induced HD mice

To determine the potential neuroprotective role of VD supplementation (500 IU/kg/day) on gait of 3-NP treated mice, we measured the distance between two successive paw prints (Fig. 3) for four weeks. No change in the stride length was observed across all the four groups of the mice respectively (Fig. 3). In comparison with Controls (Group I), HD mice (Group II) gait dynamics remained unchanged for all the respective timepoints (Group II vs Group I; 7th day, 0.90 ± 0.03 vs 0.98 ± 0.04; 14th day, 1.00 ± 0.07 vs 1.02 ± 0.06; 21st day, 0.88 ± 0.11 vs 1.09 ± 0.09; 30th day, 1.06 ± 0.05 vs 1.03 ± 0.05, $n = 4–10$, $p = 0.7$, Tukey's *post-hoc* analysis, Fig. 3A, B). Even on the 30th day, where we found a highly significant 60% decrease in the locomotion in HD mice (Fig. 2B) the gait dynamics remained unaltered between Control and HD mice (Group II vs Group I; 1.06 ± 0.05 vs 1.03 ± 0.05, $p = 0.7$; $n = 10$ each, Tukey's *post-hoc* analysis, Fig. 3B). Similarly, no effect of VD supplementation was seen in forelimb and hind-limb stride length in 3-NP treated mice (Group IV) as compared with HD mice (Group II) for entire timeline of the study (Group IV vs Group II; 7th day, 0.88 ± 0.09 vs 0.90 ± 0.03; 14th day, 0.79 ± 0.03 vs 1.00 ± 0.07; 21st day 0.88 ± 0.02 vs 0.88 ± 0.11; 30th day, 1.03 ± 0.04 vs 1.06 ± 0.05, $n = 4–10$, $p = 0.70$, Tukey's *post-hoc* analysis, Fig. 3A, B). A one-way balanced repeated measures ANOVA was conducted for the 30th day timepoint to cross check whether VD supplementation modulated gait dynamics in HD mice. No significant change in gait dynamics was observed with VD administration across all groups of mice ($F_{(3)} = 0.53$, $p = 0.66$, Fig. 3B). A power analysis done only for the 30th day gave a value of 1. This time point was chosen primarily because we found a robust effect of VD at this time point in other behavior tests (Fig. 2 and Fig. 4). Consequently, VD supplementation (either alone or in conjunction with 3-NP treatment) also did not impact the stride length performance of the mice across all time points of the present study. Our results agree with the findings of Fernaugut et al. (2002) where even a much higher cumulative dose of 3-NP (340 mg/kg) resulted in no

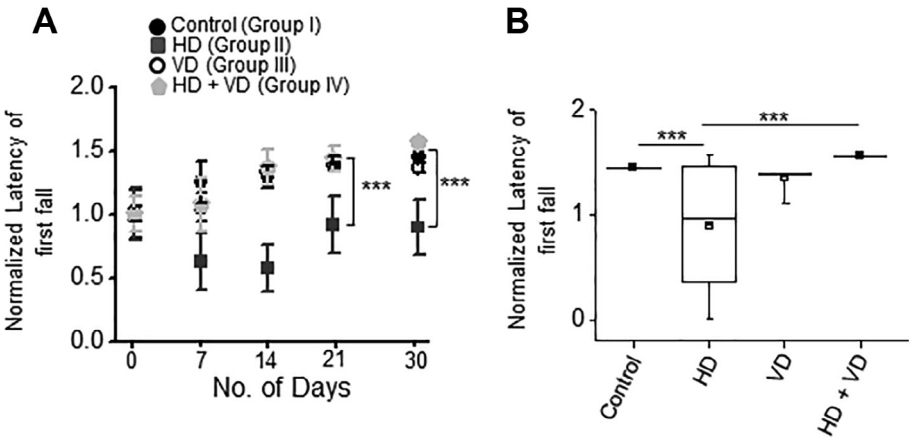

Fig. 4. Rotarod performance data depicting the beneficial effect of VD administration in HD mice. **(A)** Grip-strength of 3-NP induced HD mice (HD + VD) was significantly improved on VD supplementation ($p < 0.001$, $n = 8–9$, two-way ANOVA). All data are normalized values against the initial day for each group and is represented as mean ± SEM. **(B)** On 30th day, HD mice supplemented with VD (HD + VD) showed no latency in fall for the entire 180 s from the rotating rod, as compared HD mice ($n = 8$, $p < 0.001$, Tukey's *post-hoc* analysis). HD mice induced with 3-NP showed a significant decrease in fall latency as compared to Control (Group I) ($n = 8–9$, $p < 0.001$, Tukey's *post-hoc* analysis). Data is represented as box-and-whisker plots depicting median with first and third quartiles; shaded square is the mean for each group and whiskers represents 5th and 95th percentile values.

differences in stride length for either forelimbs and hind limbs in mice. The data suggest that since the postural gait control is regulated through reciprocal connections between the brainstem and cerebellar cortex, the obtained result may reflect that the dose of 3-NP (75 mg/kg) used in the present study did not possibly produce a significant neuronal loss in the cerebellum (Takakusaki 2017).

VD supplementation improves rotarod performance of HD mice

To test the potential effect of VD supplementation to rescue grip strength in 3-NP induced HD mice, we used the rotarod to determine the latency of first fall for the evaluation of motor coordination for four weeks (Amende et al., 2005; Rodrigues et al., 2019). We found that on the 7th day as well as on the 14th day, 3-NP injected HD mice consistently showed around a 50% reduction in fall latency when compared with the aged-matched Control animals (Group II vs Group I; 7th day; 0.63 ± 0.22 vs 1.40 ± 0.15; 14th day, 0.58 ± 0.19 vs 1.33 ± 0.08, $n = 8–9$, $p < 0.001$, two-way ANOVA followed by Tukey's *post-hoc* analysis, Fig. 4). On the 21st and 30th days, 3-NP treated mice still had a roughly 35% decrease in the latency to fall as compared to Control mice (Group II vs Group I; 21st day, 0.92 ± 0.22 vs 1.40 ± 0.05; 30th day, 0.90 ± 0.22 vs 1.45 ± 0.001, $n = 8–9$, $p < 0.001$, two-way ANOVA followed by Tukey's *post-hoc* analysis). A significant improvement in the neuromuscular coordination was observed between Group IV mice (HD + VD) and Group II mice (HD) from the 7th day onwards and continued through the 30th day (Fig. 4). Astonishingly, Group IV (HD + VD) mice showed a highly significant effect of VD supplementation on rotarod performance on the 14th day by 1.4 fold (1.37 ± 0.13), on the 21st day by 0.6-fold (1.44 ± 0.1) and on the 30th day by 0.74 fold (1.57 ± 0.001) as compared to Group II mice (HD) for the same time points (14th day, 0.58 ± 0.19; 21st day, 0.92 ± 0.22; 30th day, 0.90 ± 0.22, $n = 8–9$, $p < 0.001$, Tukey's *post-hoc* analysis, Fig. 4A, B). To our surprise VD treatment to pre-3-NP injected mice (Group IV; HD + VD) recorded no latency to fall within a total time duration of 180 s and rescued the neuromuscular coordination by 100% when compared with 3-NP induced HD mice. To rule out the possibility that VD supplementation alone showed any effect on the grip strength of mice, VD injections were carried out in a Control group (Group III). Interestingly, we found no significant difference in the latency to first fall between the Group I (Control) and the VD supplemented mice (Group III) for all time points ($p = 0.9$; Tukey's *post-hoc* analysis). Overall, two-way ANOVA showed a significant difference in the mean among all groups of mice with no interaction between the groups and day ($F_{(5)} = 4.06$, $p < 0.001$, Fig. 4), reflecting the effects of VD and 3-NP in Group II and Group IV mice. These result support our hypothesis that the VD supplementation has a robust rescue effect on neuromuscular coordination, which is sustained throughout the timeline of the study. Neuromuscular coordination is impaired in patients with Huntington's disease but how VD might affect the HD associated behavioral performance is not well described in the mouse model (Chel et al., 2013). Our data parallels the findings of Sakai et al. (2015) who showed that an oral supplementation of the VD analogue eldecalcitol (ED-71, ELD), a derivative of 1,25 $(OH)_2D_3$, for 14 days significantly improved the locomotor performance of mice. Here we used a similar dose of VD (500 IU/kg/day; 12.5 μg/kg/day) for a similar about of time (here 15 days) to explore the motor benefits of VD (cholecalciferol) in HD mice. Our findings collectively suggest that motor performance deficits observed in the 3-NP mouse model of HD get significantly reversed by VD supplementation, suggesting a neuroprotective function of VD in the striatum.

VD supplementation increases neurotrophin expression in the striatum of 3-NP induced HD mice

Alterations in the mRNA expression of the neurotrophins were analyzed in striatal tissues from all the four groups of mice, with NGF analyzed by semiquantitative PCR and BDNF by RT-PCR. RT-PCR results for BDNF expression in the striatum showed a significant change in the gene expression induced by Vitamin D_3 supplementation in HD mice ($n = 3$, $p = 0.04$, Kruskal–Wallis test, Fig. 5A). HD mice showed a significant decrease in the gene expression of BDNF as compared

to Controls (Group II vs Group I; 0.53 ± 0.06 vs 1.00 ± 0.00, $n = 3$, $p = 0.001$, unpaired sample *t*-test). VD administration after 3-NP injection robustly increased the BDNF expression in Group IV mice (3.10 ± 0.57) when compared with HD mice (Group II mice; 0.53 ± 0.06, $n = 3$, $p = 0.01$, unpaired sample *t*-test, Fig. 5A, Supplementary Table 5) reflecting that the biological effect of VD was not compromised by 3-NP induction. In addition, no significant difference in the BDNF expression was observed in the striatal tissues of Group III with respect to Group I mice (VD vs Control; 1.41 ± 0.40 vs 1.00 ± 0.00, $n = 3$, $p = 0.35$, unpaired sample *t*-test, Fig. 5A, Supplementary Table 5).

Similarly, semiquantitative PCR results showed an overall difference in the mRNA expression of NGF in all the four groups of mice ($F_{(3)} = 4.01$, $p = 0.03$, one-way ANOVA). In HD mice (Group II) the expression of NGF was downregulated by ~0.34-fold (3-NP; 0.66 ± 0.04, $n = 4$) when compared to Group I animals (Control; 1.00 ± 0.00, $n = 4$, $p = 0.001$, paired sample *t*-test, Fig. 5B). VD supplementation significantly rescued the expression of NGF in the striatum by ~0.7 fold in Group IV mice as compared with HD mice (HD + VD vs HD; 1.14 ± 0.12 vs 0.66 ± 0.04, $n = 4$, $p = 0.006$, paired sample *t*-test, Fig. 5B, C). Treatment of VD alone enhanced NGF expression by ~0.8 fold in Group III mice as compared to HD mice (VD vs HD; 1.19 ± 0.21 vs 0.66 ± 0.04, $n = 4$, $p = 0.03$, paired sample *t*-test, Fig. 5B, C) but did not significantly change NGF relative to Group I mice. These data indicate that HD mice have reduced neurotrophin expression and this is rescued by VD supplementation. The enhanced neurotrophin expression could underlie the neuroprotective effect of VD in HD mice. Our results parallel the findings of Mohamed et al. (2015) where VD treatment significantly alleviated beta-amyloid plaque expression with a concomitant elevation in the expression of neurotrophins in a rat model of Alzheimer's disease.

VD supplementation attenuates oxidative stress as reflected by the decrease in the antioxidant enzyme expression in HD mice

To observe the effect of VD supplementation on the gene expressions of antioxidant markers, we performed semiquantitative PCR in all the four groups of mice (Group I to Group IV). mRNA expressions of superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), glutathione peroxidase 4 (GpX4), and catalase (Cat) were subsequently analyzed.

(i) Superoxide dismutase1 (SOD1) and superoxide dismutase 2 (SOD2):

The effect of VD supplementation did not significantly change the gene expression of SOD1 among the four groups of mice ($F_{(4)} = 0.54$, $p = 0.71$, one-way ANOVA, Supplementary Table 6). Striatal tissue from HD mice showed no change in SOD1 mRNA expression (0.86 ± 0.42, $n = 4$) when compared with Group I (Control; 1.00 ± 0.00, $n = 4$, $p = 0.38$, paired sample *t*-test, Fig. 6A). VD administration in HD mice also showed no significant change in SOD1 expression in the striatal samples of Group IV mice (HD + VD; 1.57 ± 0.45) when compared with Group II animals (HD; 0.86 ± 0.42, $n = 4$, $p = 0.99$, paired sample *t*-test), Fig. 6A, Supplementary Table 6). VD supplementation alone did not affect SOD1 mRNA expression in Group III mice when compared with Group I (VD vs Control; 1.41 ± 0.43 vs 1.00 ± 0.00, $n = 4$, $p = 0.79$, paired sample *t*-test, Fig. 6A).

SOD2 mRNA expression also remained unchanged among all the four groups of mice either on

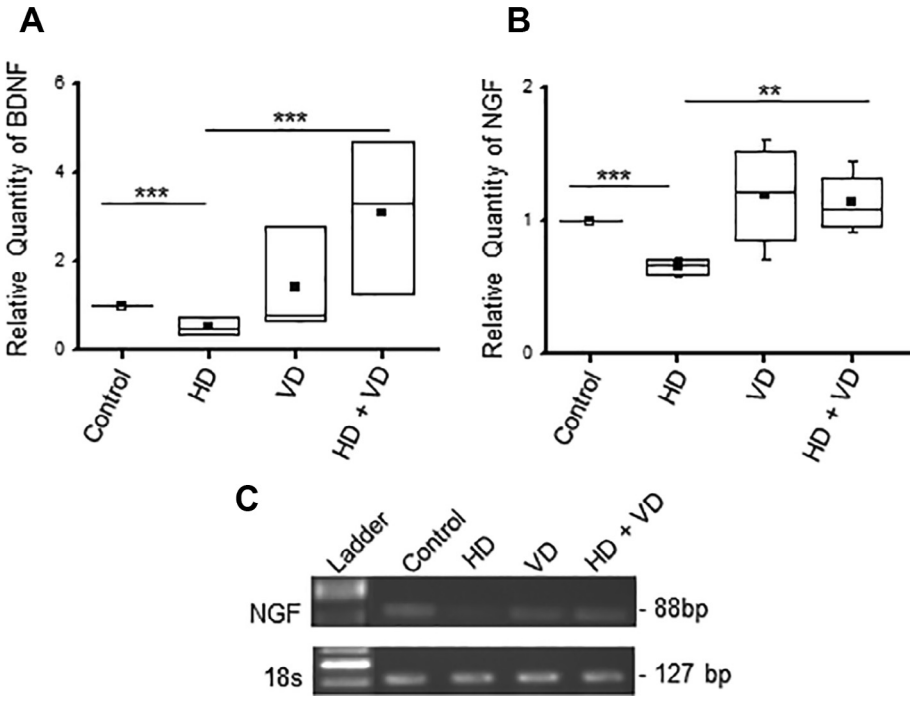

Fig. 5. mRNA expression of BDNF and NGF from the striatal tissues of mice depicting the neuroprotective effect of 500 IU/kg of VD. **(A)** Real Time – PCR results depicting robust enhancement in the mRNA expression of BDNF in the striatum of HD mice upon VD administration (HD + VD vs HD; $n = 3$, $p = 0.01$, unpaired sample *t*-test). Striatal tissue of HD mice showed a significant decrease in the gene expression of BDNF (HD vs Control; $n = 3$, $p = 0.001$, unpaired sample *t*-test). **(B)** Semiquantitative PCR results depicting VD administration rescued the mRNA expression of NGF in the striatum of 3-NP induced HD mice (HD + VD vs HD; $n = 4$, $p = 0.001$, paired sample *t*-test). NGF expression was significantly downregulated in HD mice as compared to Control (HD vs Control; $n = 4$, $p = 0.006$, paired sample *t*-test) **(C)** Representative gel images of PCR results for NGF. Data is represented as box-and-whisker plots depicting median with first and third quartiles; shaded square is the mean for each group and whiskers represents 5th and 95th percentile values.

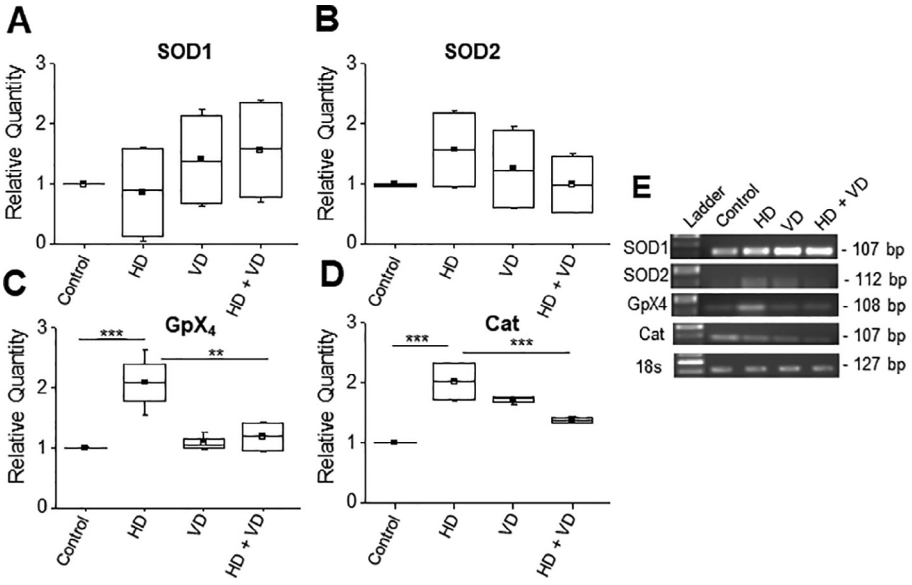

Fig. 6. mRNA expression of antioxidants from the striatal tissues of mice depicting reduced oxidative stress after VD administration in HD mice. **(A)** On 30th day, no significant change in the mRNA expression of superoxide dismutase1 (SOD1) were observed across all groups of mice (n = 4, p = 0.71, one-way ANOVA). VD induction produced no change in the striatal expression of SOD1 in Group IV mice (HD + VD) as compared to HD mice (n = 4, p = 0.99, paired sample t-test). **(B)** No significant change in the mRNA expression of superoxide dismutase 2 (SOD2) were observed across all groups of mice (n = 4, p = 0.47, one-way ANOVA). Vitamin D supplementation did not significantly change the expression of SOD2 in Group IV mice (HD + VD) as compared to HD mice (n = 4, p = 0.99, paired sample t-test). **(C)** Box and whisker plot depicting enhanced expression of glutathione peroxidase 4 (GpX4) following 3-NP injection in HD mice (HD vs Control; n = 4, p = 0.008, paired sample t-test). GpX4 expression significantly subsided upon VD administration in Group IV mice (HD + VD) as compared to HD mice (n = 4, p = 0.007, paired sample t-test). **(D)** Box and whisker plot depicting VD administration leading to decrease in catalase expression in Group IV mice (HD + VD) as compared to Group II (HD) (n = 4, p = 0.02, paired sample t-test). Striatal expression of catalases was enhanced in HD mice when compared to Control mice (n = 4, p = 0.005, paired sample t-test). **(E)** Representative gel images of PCR results for SOD1, SOD2, G_PX4 and Cat in the striatum of Control, HD, VD and HD + VD mice. Data are represented as box-and-whisker plots indicating median, first and third quartile, and 5th and 95th percentile values.

3-NP treatment or VD supplementation ($F_{(3)}$ = 0.91, p = 0.47, one-way ANOVA, Fig. 6B). SOD2 mRNA expression in HD mice was modulated by ~0.6 fold as compared to Control but did not reach significance (1.57 ± 0.35, n = 4, p = 0.90, paired sample t-test, Fig. 6B). Striatal samples from Group IV mice showed an insignificant change in SOD2 mRNA expression when compared with Group II mice (HD + VD vs HD; 0.99 ± 0.27 vs 1.57 ± 0.35, n = 4, p = 0.99, paired sample t-test, Fig. 6B, Supplementary Table 6). Also, no change in the expression of SOD2 was observe in Group III mice supplemented with only VD when compared with Group I mice (VD vs Control; 1.25 ± 0.37 vs 0.99 ± 0.01, n = 4, p = 0.54, paired sample t-test, Fig. 6B).

(ii) Glutathione peroxidase (GpX4):

On the 30th day after 3-NP induction in HD mice, an overall change in the gene expression of GpX4 in the striatal tissue was observed ($F_{(3)}$ = 14.06, p < 0.001, one-way ANOVA, Fig. 6C, Supplementary Table 6). PCR data for GpX4 revealed that 3-NP treatment caused a significant increase in the expression of GpX4 in the striatum of HD mice as compared with Group I mice (Group II vs Group I; 2.09 ± 0.22 vs 1.00 ± 0.00, n = 4, p = 0.008, paired sample t-test, Fig. 6C). mRNA expression of GpX4 in Group IV mice (HD + VD) decreased with VD administration as compared to the HD mice (Group IV vs Group II; 1.19 ± 0.11 vs 2.09 ± 0.18, n = 4, p = 0.007, paired sample t-test, Fig. 6C), to roughly control levels. Similarly, VD supplementation alone in Group III mice did not change GpX4 expression relative to Group I (VD vs Control; 1.08 ± 0.05 vs 1.00 ± 0.00, p = 0.99, paired sample t-test, Fig. 6C, Supplementary Table 6).

(iii) Catalase (Cat):

Similar results were seen with expression of the antioxidant enzyme catalase. PCR data from the 30th day post-HD induction revealed an overall change in catalase expression across all the four treatment groups ($F_{(3)}$ = 23.27, p < 0.001, one-way ANOVA, Fig. 6D, Supplementary Table 6). 3-NP injected HD mice showed a significant increase in the enzyme expression as compared with Group I mice (Group II vs Group I; 2.02 ± 0.18 vs 1.00 ± 0.00, n = 4, p = 0.005, paired sample t-test, Fig. 6D). Vitamin D_3 administration appears to reduce the oxidative stress in HD mice as seen by the decrease in catalase expression in Group IV mice (HD + VD) (Group IV vs Group II; 1.38 ± 0.03 vs 2.02 ± 0.18, n = 4, p = 0.02, paired sample t-test, Fig. 6D). VD supplementation alone in Group III mice also showed a decrease in the mRNA expression of catalases when compared to HD mice but was not significant (VD vs HD; 1.72 ± 0.03 vs 2.02 ± 0.18, p = 0.08, paired sample t-test, Fig. 6D, Supplementary Table 6). VD supplementation in Group IV mice (HD + VD) showed a decrease in the expression of antioxidants markers with a subsequent partial rescue in the body weight (supplementary Fig. 1). An overall significant difference in mean body weight was observed among all the groups of mice ($F_{(3)}$ = 5.40, p = 0.002, two-way ANOVA, supplementary Fig. 1, Supplementary Table 4). A 30% decrease in the body weight was observed by 30th day in Group II mice when compared with Group I mice (HD vs Control; 0.87 ± 0.01 vs 1.23 ± 0.05, n = 8–10, p < 0.001, paired sample t-test supplementary Fig. 1). The body weight was

significantly rescued on Vitamin D_3 supplementation in HD mice (HD + VD vs HD; 1.10 ± 0.05 vs 0.87 ± 0.01, n = 8–10, p < 0.001, paired sample t-test, supplementary Fig. 1), possibly reflecting the effect of VD in fixing oxidative stress, mitochondrial function, and muscle heath (Latham et al., 2021; G. W. Kim and Chan 2001; Chabas et al., 2013; Gueye et al., 2015).

VD supplementation increases the expression of VDR in striatum of 3-NP induced HD mice

The effect of Vitamin D supplementation on expression of the Vitamin D receptor (VDR) in the striatum was elucidated by western blot analysis ($F_{(3)}$ = 5.48, p = 0.01, one-way ANOVA, Fig. 7, Supplementary Table 7). 3-NP mediated neurodegeneration caused a significant decrease in VDR expression by ~0.54 fold in HD mice (Group II) as compared to the Control (Group II vs Group I, 0.46 ± 0.15 vs 1.00 ± 0.00, n = 4, p = 0.02, paired sample t-test, Fig. 7A). VD supplementation rescues this effect as Group IV mice (HD + VD) showed a significant increase in the expression of VDR by ~2-fold as compared to Group II (HD) mice (1.28 ± 0.26 vs 0.46 ± 0.15, n = 4; p = 0.04, paired sample t-test). An enhancement in the protein expression of VDR was observed in Group III mice, supplemented with only VD as compared to Group I mice but did not reached significance (VD vs Control; 1.52 ± 0.25 vs 1.00 ± 0.00, n = 4; p = 0.13, paired sample t-test). Our results parallel the finding of Lima et al., 2018 where VD administration enhanced the expression of VDR in the hippocampus.

DISCUSSION

In the last decade, Vitamin D_3 (1α,25-dihydroxyvitamin D_3) and its analogues have been explored for their usefulness in brain disorders. A number of studies have reported a link between low serum level of VD in patients affected by neurodegenerative and neuropsychiatric disorders like Alzheimer's Disease (AD), Parkinson's Disease (PD), Huntington's disease (HD), Schizophrenia, sleep disorders, autism, and depression (Kim et al., 2006; Chabas et al., 2013; Gueye et al., 2015; Mohamed et al., 2015; Sakai et al., 2015; Koduah et al., 2017; Morello et al., 2018; Bivona et al., 2019; Bakhtiari-Dovvombaygi et al., 2021). Under a number of neuropathological conditions, Vitamin D supplementation has shown to have a myriad of biological functions including reducing the expression of oxidative stress markers and neuro-inflammatory markers and increasing the expression of neurotrophins (Mohamed et al., 2015; Lima et al., 2018; Rodrigues et al., 2019; Bakhtiari-Dovvombaygi et al., 2021; Latham et al., 2021). Though some of the results remain inconclusive, the limited information available suggests a neuroprotective function of VD in the context of the motor dysfunction observed in Huntington's disease (HD). The goal of the present study was to explore the therapeutic potential of Vitamin D_3 (VD) in an animal model of HD induced by intraperitoneal injection of 3-nitropropionic acid (3-NP). 3-NP is a well-established toxic model causing mitochondrial dysfunction and selective loss of striatal neurons (Túnez et al., 2010; Brouillet, 2014). In this study, we used a subacute dose of 3-nitropropionic acid, a slight modification from the protocol of Amende et al. (2005). The protocol is derived from earlier studies by Fernagut et al. (2002) and Kim and Chan (2001), where 50 mg/kg of 3-NP was given for 5 days. As described by Nishino et al. (1997), a single low dose injection of 3-NP (20 mg/kg) was insufficient to induce behavioral and biochemical abnormalities in the striatum but subsequent injections caused significant striatal lesions and motor deficits. Our data show that treating 3-NP HD model mice with 500 IU/kg/day of Vitamin D_3 produces significant improvements in movement and motor performance (Fig. 2 and Fig. 4). The dose of VD was chosen based on prior studies of its neuroprotective, antidepressant, and antioxidant effect in rodent model (Chabas et al., 2013; Gueye et al., 2015; Kolla and Majagi, 2019; Rodrigues et al., 2019). These studies suggested that a dose of 500 IU/kg/day (12.5 μg/kg) of VD improved myelination and accelerated functional recovery of nerve post injury (Chabas et al., 2013). In another study, 500 IU/kg/day of VD significantly improved the locomotion performance of rodents in a spinal cord injury model that was not observed with a dose of 200 IU/kg/day (Gueye et al., 2015). Further, Rodrigues et al. (2019) demonstrated that in rodent model of sporadic dementia of Alzheimer's type, 500 IU/kg/day of VD was enough to reduce oxidative stress markers and restore cholinergic function by decreasing acetylcholine esterase activity in synaptosomes. Based on these findings, we utilized the chronic administration of 500 IU/kg/day for 15 days in order to explore its effect on motor disabilities in the 3-NP induced mouse model of HD. We also tested if any benefits were maintained over the next 15 days in the absence of continued VD administration, and our data supported that this is the case.

A study undertaken by Chel et al. (2013) suggested for the first time the importance of VD in HD by providing a link between VD deficiency and HD. In the same study,

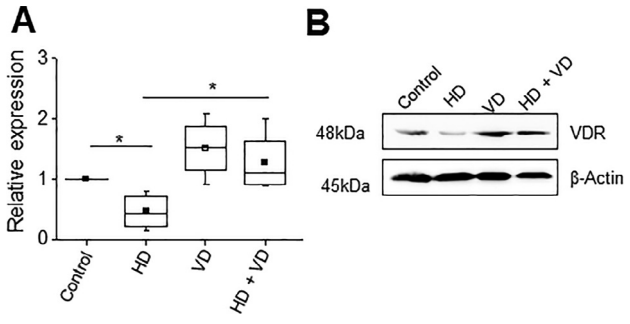

Fig. 7. Enhanced protein expression of the Vitamin D receptor (VDR) in the striatum of HD mice. **(A)** Box and whisker plot depicting the effect of VD supplementation on VDR expression in HD mice. The protein expression of VDR was significantly compromised in HD mice and reversed substantially upon VD administration (HD vs Control, n = 4, p = 0.02; HD + VD vs HD, n = 4, p = 0.04, paired sample t-test). Data are normalized against Control Data; plots indicating median, first and third quartile, and 5th and 95th percentile values. **(B)** Representative protein expression levels of VDR in the striatum of Control, HD, VD and HD + VD mice.

the author showed a positive correlation between high serum levels of 25-hydroxycholecalciferol (25(OH)VD_3 or calcidiol) levels and improvement in motor capabilities in HD patients. This was supported by the study by Xue et al. (2015), which showed that the serum concentration of Vitamin D_3 (VD) metabolite strongly influences the bioavailability of Vitamin D_3 metabolites in the brain. To produce the metabolically active form of VD (1α,25-dihy droxycholecalciferol; 1α,25-$(OH)_2$-VD_3 or calcitriol), Vitamin D_3 (VD or cholecalciferol) undergoes two hydroxylation step reactions. The first hydroxylation reaction occurs in the liver to produce 25-hydroxycholecalciferol (25(OH)VD_3 or calcidiol) and then a second hydroxylation occurs in the kidney to produce the metabolic active form of Vitamin D_3 (1α,25-dihydroxycholecalciferol; 1α,25-$(OH)_2$-VD_3 or calcitriol) (Xue et al., 2015; Bivona et al., 2019). The serum half-life of the active metabolic form of Vitamin D_3 is reported to be approximately 4–6 h while the serum half-life of calcidiol is approximately 10–21 days. The serum level of calcidiol is the most accurate and accepted method to depict VD status of an organism (Xue et al., 2015). Our results demonstrate that even in absence of systemic injection of VD (from the 15^{th} to 30^{th} day; Fig. 2 and Fig. 4), significant improvement was observed in locomotion and rotarod performance of animals over this span, especially on the 30^{th} day. These data corroborate the findings of Xue et al. (2015), suggesting that the serum levels of VD influence brain VD metabolite levels and impact the motor capabilities of HD model animals, neurotrophin levels and oxidative stress.

The rescue effect of VD administration in 3-NP induced HD mice were tested on movement impairment, stride length and grip strength to evaluate the motor coordination of the animals (Beal et al., 1993; Baydyuk and Xu, 2014). Group II mice (3-NP induced) showed a reduction in their latency of fall on the rotarod, whereas on 30^{th} day, Group IV mice (HD + VD) rescued neuromuscular coordination and showed no latency of first fall within a total time duration of 180 seconds as shown in Fig. 4. Neuromuscular coordination is known to be impaired in patients with Huntington's disease but how VD affects this behavior performance in HD have not been described in mouse model (Chel et al., 2013). The findings of the present study suggest that the motor performance deficits observed in the 3-NP model of HD were significantly reversed by VD supplementation, suggesting a neuroprotective function of VD in the striatum. We observed no variability in the gait dynamics across all the four groups (Group I-IV) over a month's time as shown in Fig. 3, possibly reflecting that the dose of 3-NP (75 mg/kg) used in the present study did not produce neuronal loss in the cerebellum (Takakusaki, 2017). Hence, no rescue effect of VD was observed in 3-NP injected HD mice (Fig. 3). Changes in gait or postural control could occur with different doses or schedules of neurotoxin (3-NP) injection than those undertaken in the present study.

The enhancement in locomotory and rotarod performances of HD mice post injected with VD (Group IV; HD + VD) was accompanied with an increase in the expression of brain derived neurotrophic factor (BDNF), nerve-growth factor (NGF), and the Vitamin D receptor (VDR) (Fig. 5 and Fig. 7). Previous studies have found that VD mediates an increase in the expression of Vitamin D receptor (VDR), tyrosine hydroxylase (TH), the dopamine transporter (DAT), and brain derived neurotrophic factors (BDNF) (Nimitphong and Holick, 2011). VD mediates its biological effect via VDR by acting as transcriptional regulator for some important neurotrophins in the brain like NGF and BDNF (Johri and Beal, 2012; Taniura et al., 2006; Allen et al., 2013; Zuccato and Cattaneo, 2007; Silva et al., 2015; Bayo-Olugbami et al., 2020; Nadimi et al., 2020). To test some of these previously reported targets, semi-quantitative PCR and RT-PCR was carried out to explore VD-induced gene expression of neurotrophins in Control and 3-NP treated group of mice. In agreement with earlier literature reports, we found a significantly decreased expression of BDNF and NGF in 3-NP injected HD mice, but this profoundly augmented in the Group IV mice (HD + VD) with supplementation of VD (500 IU/kg) (Fig. 5A, B) (Saporito et al., 1994; Pérez-Navarro et al., 2000; Chabas et al., 2013; Gueye et al., 2015; Silva et al., 2015; Bayo-Olugbami et al., 2020; Nadimi et al., 2020). Numerous studies have highlighted the importance of neurotrophic factors like BDNF and NGF as potential therapeutics for neurodegenerative diseases such as Parkinson's, Alzheimer's, and Huntington's diseases (Kim et al., 2006; Sanchez et al., 2009; Mohamed et al., 2015; Lima et al., 2018; AlJohri et al., 2019; Rodrigues et al., 2019). In particular, *in-vivo* and *in-vitro* findings from Zuccato et al. (2001) suggest that restoring BDNF production in cortical neurons during HD could restore the survival signal required by the dying striatal neurons (Zuccato and Cattaneo, 2007). The same study also provided evidence using genetic models of HD that mutant huntingtin profoundly diminished the cortical production of BDNF. Further, the work conducted by Pérez-Navarro et al. (2000) suggests BDNF to be the most effective factor in preventing the loss of striatal neurons in HD. Our data demonstrate that the gene expression of BDNF and NGF was significantly compromised in 3-NP induced HD mice (Group II) and was substantially reversed upon VD administration in Group IV mice. This result suggests a direct therapeutic benefit of VD in combating 3-NP induced striatal neurodegeneration via BDNF and NGF in the striatum (Fig. 5). NGF and BDNF are established candidates for combating the death of neurons observed in a range of neurodegenerative disorders (Zuccato and Cattaneo, 2007; Gil-Mohapel, 2012; Allen et al., 2013). VD supplementation possibly enhances the survival signals from neurotrophins to reduce neurodegeneration and combat striatal neuronal loss as observed in the rat model of AD (Mohamed et al., 2015). These results indicate that VD could alleviate behavior deficits in 3-NP induced HD mice via enhancement in neurotrophins expression in the striatum.

The enhancement in the production of neurotrophins like BDNF could act to reduce oxidative stress in neurodegenerative diseases including HD (Taniura et al., 2006; Allen et al., 2013; Takakusaki, 2017; Paul and Snyder, 2019; Bakhtiari-Dovvombaygi et al., 2021).

Oxidative stress markers allow assessment of the status of the biological samples where it measures the capacity of the system to scavenge free radicals. To control the intracellular redox balance, cells have evolved a highly complex ROS scavenging network. Previous studies on the antioxidant role of VD have been controversial as some studies did not support an antioxidant function for VD and other studies observed an up-regulation of the antioxidant markers (Loscalzo, 2008; Seiler et al., 2008; Tagliaferri et al., 2019). To determine whether, in our model, similar pathways are activated we checked different antioxidant enzymes marker genes. The glutathione (GSH)-dependent enzymatic system is one of most important ROS balancing units that regulates cell survival against oxidative damage. GSH contributes to the maintenance of the intracellular redox environment either by disulfide-exchange reactions with oxidized proteins or by acting as a reducing agent for glutathione peroxidases. Out of seven Glutathione peroxidases of mammals, GpX4 is particularly important due to its critical role in determining the cell membrane redox state. Increased expression of GpX4 indicates lipid based oxidative stress (Tagliaferri et al., 2019). In Group II (HD animals) we found a significant increase in expression of GpX4 indicating higher oxidative stress and this was attenuated upon supplementation with VD (Group IV, See Fig. 6). Catalase is one of the crucial antioxidant enzymes that mitigates oxidative stress by destroying cellular hydrogen peroxide to produce water and oxygen (Loscalzo, 2008; Seiler et al., 2008; Tagliaferri et al., 2019). Supporting the GpX4 expression data which indicates higher oxidative stress, HD (Group II) animals showed increased expression of catalase, which was again diminished by VD supplementation. This suggests that VD supplementation reduces oxidative stress and leading to the subsequent downregulation of antioxidant enzymes. We could not find the significant differences in SOD1 and SOD2 expression possibly because its activation depends on very specific ROS species.

The antioxidant effect of VD supplementation in HD mice was accompanied by enhancement in the protein expression of Vitamin D receptor (VDR) in the striatum (Fig. 7). Previous studies have reported that the biological activity of VD happens via upregulation of VDR in other neurodegenerative diseases like AD and PD (Mohamed et al., 2015; Lima et al., 2018). Therefore, the protein expression of VDR was analyzed in Group IV mice (HD + VD) pre-injected with 3-NP. On the 30th day, a robust expression of VDR by ~2 fold was observed in HD mice supplemented with VD (Fig. 7). HD mice (Group II) showed a significant decrease by ~0.54 fold in the VDR expression as compared to Control (Group I). This enhanced VDR expression could help in attenuating the toxic effect of 3-NP thereby reducing antioxidant stress markers and increasing neurotrophins expression in Group IV mice. The improvement in motor performance observed in HD mice could also occur due to increased Vitamin D receptor signaling at the neuromuscular junction as seen previously (Sakai et al., 2015). Additional contribution of VD supplementation to the neuroprotective role of the cholinergic system may also be a factor, as has been seen in AD (Rodrigues et al., 2019). A reduction in cholinergic signaling may occur in HD due to aberrant kinase signaling as studies have shown that protein kinases are known modulators of cholinergic receptors expression and function (Komal et al., 2014; 2015). Previous studies have demonstrated that Vitamin D receptor (VDR) signaling alleviates oxidative stress and increases production of neurotrophins like BDNF (Bakhtiari-Dovvombaygi et al., 2021; Xu and Liang, 2021). It is likely that in our study, the rescue effect of VD observed in behavior tasks involves the VD-VDR signal transduction pathway, potentiating survival signals via neurotrophins and decreasing oxidative stress, which in turn downregulates antioxidant stress markers (Fig. 8). It is known that VDR signaling is vital for mitochondrial integrity, combats ER stress and strengthens skeletal muscle activity at neuromuscular junction (Baydyuk and Xu, 2014; Sakai et al., 2015; Bakhtiari-Dovvombaygi et al., 2021; Xu and Liang, 2021; Maity et al., 2022). In summary, our data suggests that Vitamin D_3 mediates a neuroprotective effect in the striatum via enhancement in the expression of Vitamin D receptor (VDR) and vital neurotrophins, like BDNF and NGF, crucial for survival signals in HD.

3-NP induction also significantly decreased the body weight of HD mice (Group II) by ~0.3-fold as previously reported by Kumar et al. (2009), which was reversed upon VD supplementation by the end of 30 days (HD + VD; Group IV, supplementary Fig. 1). 3-NP is known to cause mitochondrial dysfunction similar to what is demonstrated

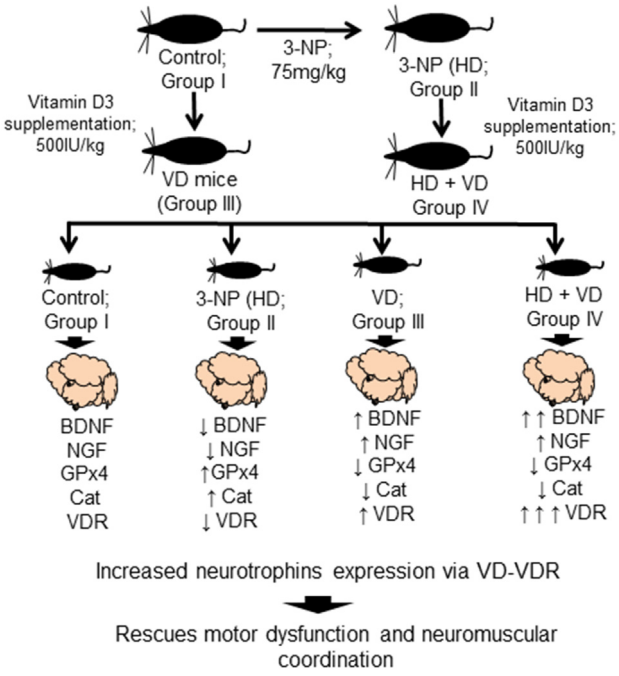

Fig. 8. Restorative effects of Vitamin D_3 (VD) in Huntington's disease (HD). VD supplementation enhances Vitamin D receptor (VDR) in the striatum with concomitant increase in the expression of neurotrophins namely, brain derived neurotrophic factor (BDNF) and nerve growth factor (NGF) in the striatum of HD mice (Group IV). The increased gene expression of neurotrophins is proposed to occur through biological effects of VD on Vitamin D receptor (VDR), potentiating antioxidant and neuroprotective benefits of VD on motor activity.

in a genetic model of HD (Brouillet et al., 2005; She et al., 2011). Our gene expression and protein expression data reflect that at this time point (30th day) there is a significant enhancement in the Vitamin D receptor expression (VDR) in the striatum with a concomitant increase in expression of BDNF and NGF. The therapeutic action of VD possibly involves an increase in the expression of VDR in the skeletal muscles and increasing muscle mass, and increasing body weight. This also suggests that VD may be rescuing energy impairments and mitochondrial dysfunction through upregulation of VDR and possibly could be one of the reasons why we see an enhancement in the weight in Group IV mice (Wong et al., 2009; Latham et al., 2021). Overall, our results are novel in determining the long-lasting effect of VD on striatal functions in HD, and reflecting the strong effect of this neurosteroid in combating motor dysfunction via enhancement in survival signal by BDNF and NGF (Fig. 5).

It must be noted that our study had some limitations in that we did not perform histopathological studies to determine the effect of VD on cell death in striatal neurons. Further, the 3-NP cytotoxic model is useful model for mimicking the pathophysiological symptoms of HD, but does not involve mHTT itself. In our study, HD mice showed a significant molecular change in VDR levels, antioxidant stress markers and neurotrophin expression, which are known to underlie HD pathogenesis and have been observed in transgenic models of the disorder (Vis et al., 1999; Zuccato et al., 2001; Brouillet et al., 2005; Gil and Rego, 2008; Gil-Mohapel, 2012; Brouillet, 2014). Our findings showed that VD is a promising agent for delaying or even restoring motor dysfunction. It is evident from our study and others that VD supplementation possibly involves a diversity of mechanisms for its beneficial effect in HD (Taniura et al., 2006; Bankole et al., 2015; Sakai et al., 2015; Xu and Liang, 2021). VD supplementation has proved to be effective in reversing motor deficits and neurotrophins levels in the 3-NP induced mouse model of HD. It could be considered as promising agents for the development of new therapeutics for neurodegenerative disorders including HD. However, it will remain critical to replicate our findings on neuroprotective role of VD supplementation in transgenic animal models.

CONTRIBUTION OF THE AUTHORS

MSKV, VK, PR, SM and PK designed, conducted and analyzed the experiments. MSKV, KV, PR, SM, SYY and DS wrote portions of the manuscript. MSKV, KV, SM and PK revised all the experiments of the paper and statistical analysis. PK designed and directed the experiments, conducted and analyzed some experiments, wrote and revised the manuscript. PK, SM and DS performed the final edit of the manuscript.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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APPENDIX A. SUPPLEMENTARY DATA

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuroscience.2022.03.039.

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Research paper

Unprecedented effect of vitamin D3 on T-cell receptor beta subunit and alpha7 nicotinic acetylcholine receptor expression in a 3-nitropropionic acid induced mouse model of Huntington's disease

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ABSTRACT

Introduction: 3-NP induction in rodent models has been shown to induce selective neurodegeneration in the striatum followed by the cortex (Brouillet, 2014). However, it remains unclear whether, under such a neurotoxic condition, characterized by neuroinflammation and oxidative stress, the gene expression of the immune resident protein, T-cell receptor beta subunit (TCR-β), α7 nicotinic acetylcholine receptor (α7 nAChRs), the nuclear factor kappa B (NF-κB), inflammatory cytokines (TNF-α and IL-6), and antioxidants (Cat and GpX4) get modulated on Vitamin D3 (VD) supplementation in the central nervous system.

Methods: In the present study, real-time polymerase chain reaction (RT-PCR) was performed to study the expression of respective genes. Male C57BL/6 mice (8–12 weeks) were divided into four groups namely, **Group I:** Control (saline); **Group II:** 3-NP induction via i.p (HD); **Group III**: Vitamin D3 (VD) and **Group IV:** (HD + VD) (Manjari et al., 2022).

Results: On administration of 500IU/kg/day of VD, HD mice showed a significant reduction in the gene expression of the immune receptor, TCR-β subunit, nuclear factor kappa B (NF-κB), inflammatory cytokines, and key antioxidants, followed by a decrease in the acetylcholinesterase activity.

Conclusion: A novel neuroprotective effect of VD in HD is demonstrated by combating the immune receptor, TCR-β gene expression, antioxidant markers, and inflammatory cytokines. In addition, HD mice on VD administration for 0–15 days showed an enhancement in cholinergic signaling with restoration in α7 nAChRs mRNA and protein expression in the striatum and cortex.

1. Introduction

One of the breakthroughs in the field of immune-neuronal interaction came 35 years ago when neuroscientists discovered the neuronal role of cytokine, interleukin-1 (IL-1) in the modulation of neurotransmitters release and explored its contribution toward immune-brain interaction (Kabiersch et al., 1988; Spadaro and Dunn, 1990). Thereafter, rapid advances were made in discovering the expression of immune molecules and receptors in the brain originally thought to be expressed only in the immune system. Immune proteins like major histocompatibility complex – I (MHC-I), β2 microglobulin (a co-subunit of MHC-I), and its potential binding partner CD3ζ (a protein complexed to receptors for MHC-I) were found to be expressed in neurons (Baudouin et al., 2008; Shatz, 2009; Komal et al., 2022). In addition to MHC-I, a

Abbreviations: VD, Vitamin D3; HD, Huntington's disease; 3-NP, 3-nitropropionic acid; i.p, Intraperitoneal; ACh, Acetylcholine; BDNF, Brain-derived neurotrophic factor; NGF, Nerve-growth factor; VDR, Vitamin D receptor; Cat, Catalases; GpX4, Glutathione peroxidases; α7 nAChRs, alpha7 nicotinic acetylcholine receptors; MHC-I, Major histocompatibility complex– I; MSN, Medium spiny neurons; GABA, γ-aminobutyric acid; *Htt*, Huntingtin gene; mHTT, mutant Huntingtin protein; cDNA, Complementary DNA; RT-PCR, Real-Time polymerase chain reaction; ANOVA, Analysis of variance; AD, Alzheimer's disease; PD, Parkinson's disease; 1α, 25 $(OH)_2$VD3, 1α, 25-dihydroxy vitamin D3 or calcitriol; 25OHVD3, 25-hydroxyvitamin D3 or calcidiol; ROS, reactive oxygen species; SEM, standard error of the mean.

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study undertaken by Komal et al. (2014) reflected a possible effect of T-cell receptor activation (TCR) on α7 nicotinic acetylcholine receptor expression and function in the murine cortex. However, how immune resident protein, T-cell receptor beta subunit (TCR-β) expression in the central nervous system gets modulated under a neuropathological condition like those observed in Huntington's disease (HD) remains unexplored.

Huntington's disease (HD) is a progressive, fatal, neurodegenerative disorder characterized by neuronal loss predominantly in the striatum, followed by the cortical region of the brain (Gil and Rego, 2008). Neuronal death results in motor, cognitive, and working memory impairments typically associated with the disease pathology (Gil and Rego, 2008). Some of the neurotoxic conditions responsible for neuronal loss in the striatum and the cortex as seen in HD include enhanced neuroinflammation, increased oxidative stress, decreased neurotrophins production, and mitochondrial dysfunction (Cherubini et al., 2020; Maity et al., 2022; Rekatsina et al., 2020; Zuccato and Cattaneo, 2007). 3-nitropropionic acid (3-NP) induction in mice causes selective neuronal degeneration in the caudate and putamen of basal ganglia circuitry and recapitulates a wide range of neuropathological symptoms of HD (Brouillet, 2014). 3-NP is an irreversible inhibitor of succinate dehydrogenase and is a well-known toxin-induced model of HD (Kim et al., 2003). 3-NP injections in rodents have also been shown to cause neuroinflammation and neurochemical alteration due to increased oxidative stress (Ahuja et al., 2008). In this regard, an antioxidant effect of Vitamin D3 (VD; cholecalciferol) at a dose of 500IU/kg/day was recently shown to significantly rescue motor dysfunction in a 3-NP induced mouse model of HD (Manjari et al., 2022). VD administration also caused an enhancement in the gene expression of neurotrophins like nerve-growth factor (NGF) and brain-derived neurotrophic factor (BDNF) in the striatum (Manjari et al., 2022).

There are shreds of evidence that Vitamin D3 (VD) mediates its biological effect by binding with the Vitamin D receptor (VDR) and combats neuronal loss across a range of neuropsychiatric illnesses (AlJohri et al., 2019; Bakhtiari-Dovvombaygi et al., 2021; Buell and Dawson-Hughes, 2008; Chabas et al., 2013; Nimitphong and Holick, 2011; Rodrigues et al., 2019). Nonetheless, under such neuropathological conditions, as observed across a multitude of neurological disorders like Alzheimer's disease (AD), Parkinson's disease (PD), schizophrenia (SCZ), and Huntington's disease (HD), impairment in cholinergic neurotransmissions are also discovered where specific activation of α7 nicotinic acetylcholine receptors (α7 nAChRs) have been shown to exhibit neuroprotective benefits (Caton et al., 2020; D'Angelo et al., 2021; Egea et al., 2015; El Nebrisi et al., 2020; Foucault-Fruchard et al., 2017, 2018; Hoskin et al., 2019; Marder, 2016; Quik et al., 2015; Tata et al., 2014; Zhao et al., 2021). However, the impact of Vitamin D3 (VD) supplementation on the neuronal gene expression of TCR-β subunit receptor and α7 nAChRs in HD remains largely unexplored. Also, 3-NP mediated increase in oxidative stress and its effect on acetylcholinesterase (AChE) activity in HD remains to be elucidated.

In the present study, we show that VD administration in HD mice preinjected with 3-NP significantly decreases the gene expression of TCR-β immune receptor and antioxidants like catalase (Cat), and glutathione peroxidase (GpX4) together with a concomitant reduction in the acetylcholinesterase activity in the cortex and striatal brain regions. No significant difference was observed between Group I (control mice) and Group III (mice supplemented only with VD), further supporting the present hypothesis that VD neuroprotective benefits were observed only when neurons were subjected to neurodegeneration on 3-NP administration. Overall in the present work, we primarily show an anticholinesterase activity of VD and its positive effect on α7 nicotinic acetylcholine receptor mRNA and protein expression together with a detrimental effect on the gene expression of the TCR-β subunit in Huntington's disease (HD).

2. Experimental procedures

2.1. Animal procurement

Ten to twelve weeks old male C57BL/6 mice (average weight; 26 ± 3 g) were acquired from Sainath Agencies, Hyderabad, India. Animals were group-housed (2 mice per cage) with ad libitum access to food and water. They were kept in a 12 h light/12 h dark cycle at 25 ± 2 °C. All the animal experiments were carried out with the approval of the institutional animal ethics committee (IAEC), BITS - Pilani, Hyderabad. All efforts were made to minimize the number of animals used and their suffering.

2.2. Study design

Mice were acclimatized for twelve days and were then randomly divided into 4 experimental groups (Group I to Group IV). Intraperitoneal injections (i.p) of 3-nitropropionic acid (3-NP) and/or Vitamin D3 (VD or cholecalciferol) were given as described previously (Manjari et al., 2022). Briefly, 3-NP was given thrice at a dose of 25 mg/kg, every 12 h, for a total cumulative dose of 75 mg/kg. Intraperitoneal injections (i.p) of VD were undertaken at a dose of 500IU/kg/day from day 1 to day 15 (Fig. 1; Manjari et al., 2022).

2.3. Experimental design

The mice were randomly divided into four experimental groups for biochemical assays (Fig. 1).

a. **Group I:** Control group mice (C57BL/6) injected with 1X saline.
b. **Group II:** 3-NP induced mice by i.p. injection (3-NP; 75 mg/kg) without VD-treatment (HD).
c. **Group III:** Mice injected solely with 500IU/kg/day of Vitamin D3 (VD) for 15 days.
d. **Group IV:** Post-intraperitoneal injection of 500IU/kg/day of VD to 3-NP (75 mg/kg) pre-injected mice for 15 days (HD + VD).

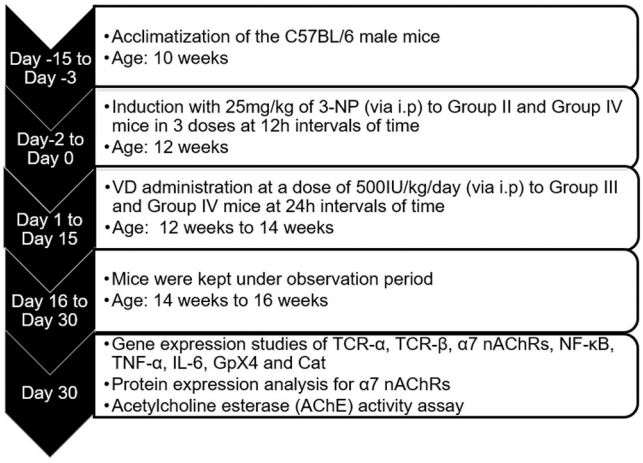

Fig. 1. Timeline and design for the study. C57BL/6 male mice at the age of ten to twelve weeks were undertaken in the present study. Mice were separated into four different groups. Group II and Group IV mice were injected (i.p) with 3-nitropropionic acid (3-NP) at 25 mg/kg dose at 12 h intervals of time (cumulative dose of 75 mg/kg; Manjari et al., 2022). Vitamin D3 (VD; 500IU/kg/day) was supplemented in Group III mice (VD only) and after post-injection of 3-NP to Group IV mice (HD + VD) for 15 days i.e. from Day 1 – Day 15. Mice were kept under observation from Day 1 to Day 30. On the 30th day, mice were sacrificed and the cortical and striatal brain tissue samples were extracted for gene and protein expression analysis.

2.4. Drugs and reagents

2.4.1. Cholecalciferol (Vitamin D3; VD)

was purchased from Sigma-Aldrich, India (Cat No: C9756) and dissolved in 1% ethanol (diluted with sterile saline) on the day of injection (Mohamed et al., 2015). Mice were administered with 500IU/kg (12.5 μg/kg/day) i.p. of VD as reported previously (Manjari et al., 2022). VD administration was undertaken in Group III mice (only VD) and Group IV mice (HD+VD).

2.4.2. 3-nitropropionic acid (3-NP)

was purchased from Sigma-Aldrich, India (Cat No: N22908). Stock solutions of 3-NP (3 mg/mL) were prepared in 0.1 M phosphate-buffered saline solution and were injected intraperitoneally at 25 mg/kg (3-NP; a cumulative dose of 75 mg/kg) thrice at 12 h intervals to respective groups of mice as described previously (Manjari et al., 2022). Controls were treated with three doses of 1X saline at 12 h intervals.

2.5. RNA isolation and cDNA preparation

On the 30th day, mice from all four groups (i.e Group I to Group IV) were anesthetized using isoflurane (Rx, NoB506) and immediately decapitated for the extraction of cortical and striatal brain tissue samples. The respective brain tissue sample was placed into 1 mL of RNAiso PLUS (Takara Bio), sonicated on ice, and centrifuged after the addition of 200 μl of chloroform for 30 min at 12,000 g at 4 °C (Eppendorf Refrigerated centrifuge, 542R). The isolation of the aqueous phase was followed by the addition of an equal volume of isopropanol (Hi-Media Laboratories, Molecular biology grade, India), followed by overnight incubation at − 20 °C. Sample washing was preceded with centrifugation at 12,000 g for 30 min at 4 °C, followed by washing with 70% ice-cold ethanol. The obtained pellet was resuspended in nuclease-free water. DNase-treated samples (EN052, Thermo Scientific™, USA) were made up to 400 μl using nuclease-free water, followed by sample purification using 1/10th volume of 3 M sodium acetate and 2X volume of phenol: chloroform: isoamyl alcohol (Sisco Research Laboratories Pvt. Ltd., India) and centrifuged for 2 min at maximum speed at 4 °C. The total concentration of purified RNA was estimated by the Nanodrop spectrophotometer (Nanodrop, Thermo Fisher Scientific, USA). An equal amount of RNA from each group was used to reverse transcribe complementary DNA (cDNA) with the help of the Verso cDNA synthesis kit (Cat No: AB1453A, Thermo Scientific™, USA) as per the manufacturer's instruction. Briefly, 500 ng of purified RNA was taken from each group for cDNA synthesis with the following reaction conditions: 42 °C for 1 h followed by 95 °C for 2 min. The obtained cDNA was used for real-time polymerase chain reaction (RT-PCR). The expression of targeted genes was normalized to 18 S RNA. All primers are listed in Table 1.

2.6. Primer design

Primers for all genes were designed using a multitude of in-silico approaches involving various bioinformatics tools. The cDNA sequences for each gene were retrieved from the Ensembl genome browser (https://asia.ensembl.org/index.html). Primer for the TCR-β subunit was directed towards the constant region as described previously (Syken and Shatz, 2003; Table 1).

2.7. Analysis of gene expression for TCR-β, α7 nAChRs, NF-κB, TNF-α, IL-6, and antioxidants by real-time polymerase chain reaction (RT-PCR)

The sequences of the immune receptor, TCR-alpha (TCR-α), TCR-beta (TCR-β), α7 nicotinic acetylcholine receptor (α7 nAChRs), nuclear factor-kappa B (NF-κB), tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6) and antioxidant marker genes (Cat and GpX4) of the mouse genome were obtained from NCBI. The sequences were deposited in the IDT primer quest tool to get the most suitable primer for gene analysis. All the genes, primer sequences, and amplicon sizes are listed in Table 1. The gene expression among the four groups of mice was assessed by RT-PCR in a CFX96 Touch Real-time PCR system (BioRad) using the GoTaq qPCR SYBR master mix (Cat No #A6001, Promega Corporation). The reaction mixture was prepared according to the manufacturer's protocol using ~12 ng of the cDNA template. Relative gene expression was quantified using the ΔCT method with respective primers (Table 1) and normalized to 18 s (forward 5'-ACGGAAGGGCACCACCAGGA-3'; reverse 5'-CACCACCACCCACGGAATCG-3'). We used the ΔΔCT method to determine the fold changes in the expression of TCR-β, α7 nAChRs, NF-κB, TNF-α, IL-6, and oxidative stress markers (Livak and Schmittgen, 2001). Briefly, the threshold cycle (Ct) was extracted using Bio-Rad CFX Manager 3.1 software, and relative gene expression was calculated as follows: fold change = 2^-ΔΔCt, where ΔCt (cycle difference) = Ct (target gene) – Ct (control gene) and ΔΔCt = ΔCt (treated condition) - ΔCt (control condition) (Livak and Schmittgen, 2001).

2.8. Acetylcholinesterase (AChE) activity assay

The acetylcholinesterase (AChE) activity was assayed using Amplex® Red Acetylcholine/Acetylcholinesterase Kit (A-12217; Invitrogen) essentially following instructions as directed by the manufacturer. In the assay, AChE activity was assessed indirectly with the help of Amplex Red, a highly sensitive dye for horseradish peroxidase (HRP). In the initial step, AChE transforms acetylcholine into choline and acts as a substrate for the choline oxidase enzyme that converts choline to betaine

Table 1
Sequence of primers used in RT - PCR studies.

Gene	Orientation	Sequence of primers (5' to 3')	Amplicon size (bp)
18 s	Forward	ACGGAAGGGCACCACCAGGA	127
	Reverse	CACCACCACCCACGGAATCG	
TCR-α	Forward	CAAGTGACCCTTTCAGAAGATGA	106
	Reverse	GTGGACCTTGTCCAGGATATTG	
TCR- β	Forward	GTGAATGGCAAGGAGGTCCA	111
	Reverse	CCAGAAGGTAGCAGAGACCC	
α7 nAChRs	Forward	GTACAAGGAGCTGGTCAAGAA	94
	Reverse	CAGGAGACTCAGGGAGAAGTA	
GPx4	Forward	GCCCAATACCACAACAGTAGA	108
	Reverse	CCTGAACCACAGCGATGAA	
Cat	Forward	AATTGCCTCCACACCTTCAC	107
	Reverse	TCACCAAGCTGCTCATCAAC	
TNF-α	Forward	CTACCTTGTTGCCTCCTCTTT	116
	Reverse	GAGCAGAGGTTCAGTGATGTAG	
IL-6	Forward	GGGATGTCTGTAGCTCATTCTG	101
	Reverse	AACTGGATGGAAGTCTCTTGC	
NF-κB	Forward	GGAACAGGTGGGATGTTGCT	187
	Reverse	GACTAAACTCCCCCTGATTCTGAAG	

and H_2O_2. Following this step, H_2O_2 reacted at a ratio of 1:1 with Amplex red to produce the fluorescent product resorufin, which in turn was measured using a fluorescent plate reader (Spiromax, USA). To analyze AChE activity, the reaction was initiated using a 100 µl working solution (50 µM acetylcholine, 200 µM Amplex Red reagent, 0.1 U/mL choline oxidase, and 1 U/mL horseradish peroxidase [HRP]) which was added to 100 µl of the brain tissue sample from each respective group of mice. After 30 min of incubation at room temperature, the fluorescence intensity was measured at 590 nm emission wavelengths. The enzyme activity was calculated using AChE standard curve and data is represented as mU/mg protein after subtraction of the background fluorescent value for each sample fluorescent value (Fig. 4).

2.9. Protein quantification and western blotting

On the 30th day, cortical and striatal brain tissue was extracted from all four groups of mice. The tissue was homogenized in the lysis buffer (150 mM sodium chloride, 1.0 % TritonX-100, 0.5 % sodium dodecyl sulfate, and 50 mM Tris, pH 8.0). The protein concentration was determined using a Bradford protein assay kit (Bio-Rad, USA). We loaded equal amounts of protein (50 µg) run in a 12 % gel, and then transferred to PVDF (Pall Corporation) membrane through a trans blot wet transfer system (Bio-Rad). The membrane was blocked using 5 % BSA and incubated with respective primary and secondary antibodies for α7 nAChRs mouse mAb (CHRNA7, 1:500, #MA5–31691, Thermo Fischer); Anti-mouse IgG-HRP-linked antibody (1:5000, AB_10015289, Jackson ImmunoResearch Laboratories). Membranes stained with ponceau (ML045, Himedia) were used as a control for normalization. The signal intensities of the bands were captured using the fusion pulse gel documentation system (Eppendorf, USA). ImageJ software was used to quantify the band intensities.

2.10. Statistical analysis

Experimental data are represented as normalized values w.r.t to control. Data in the figures are represented as box and whisker plots depicting the median with interquartile range; (central line: median; 25th and 75th quartiles; whiskers: 5th-95th percentile values) to illustrate the distribution of normalized values for each respective group of mice (Group I to Group IV). Group data in the text are presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted using one-way ANOVA followed by either post hoc multiple pairwise analysis using Tukey's HSD tests or paired sample t-test. $p < 0.05$ was set as threshold of significance ($^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$). All the data is displayed using Origin 8.1.

3. Results

3.1. Vitamin D3 supplementation decreases TCR-β subunit expression in the cortex and striatum of HD mice

To explore the chronic effect of VD on the immune receptor, TCR-β subunit mRNA expression, RT-PCR was performed on the cortical and striatal brain tissue samples extracted on the 30th day from all four groups of mice (Group I-Group IV). We found an overall significant change in TCR-β expression among all four groups of mice ($p = 0.004$; one-way ANOVA). HD mice (Group II) injected with a cumulative dose of 75 mg/kg of 3-NP showed profound enhancement ~2-fold in the gene expression of the TCR-β subunit in the cortex when compared to that of control mice (Group II vs Group I; 3.16 ± 0.32 vs 1.00 ± 0.00, n = 6, $p = 0.009$, paired sample t-test; Fig. 2A). On the 30th day, post administration of 500IU/kg of Vitamin D3 (VD) in HD mice significantly subsided the gene expression of the immune receptor, TCR-β subunit in comparison to HD mice preinjected with only 3-NP (Group IV vs Group II; 1.06 ± 0.15 vs 3.16 ± 0.32, n = 6, $p = 0.02$, paired sample t-test; Fig. 2A).

Similarly, a comparable trend of the VD effect was observed from the striatal brain tissue samples of all four groups of mice ($p < 0.001$, one-way ANOVA). The expression of TCR-β in HD mice was upregulated by ~3-fold (3-NP) when compared to the control mice (Group II vs Group I; 4.02 ± 0.52 vs 1.00 ± 0.00, n = 10, $p = 0.005$, paired sample t-test, Fig. 2B). VD supplementation significantly decreased the expression of TCR-β in the striatum of 3-NP injected mice (HD + VD) as compared with HD mice (Group IV vs Group II; 1.08 ± 0.07 vs 4.02 ± 0.52, n = 10, $p = 0.008$, paired sample t-test, Fig. 2B). Overall, these data represent that VD modulates the gene expression of the immune receptor, TCR-β under neuropathological conditions induced by 3-NP.

3.2. Vitamin D supplementation rescues the protein and mRNA expression of α7 nAChRs in the cortex and striatum of HD mice

The effect of Vitamin D supplementation on the protein expression of the α7 nicotinic acetylcholine receptor (α7 nAChRs) in the cortex was elucidated by western blot analysis. 3-NP mediated neurodegeneration caused a significant decrease in the α7 nAChRs protein expression in HD mice (Group II) as compared to the control mice (Group II vs Group I, 0.24 ± 0.08 vs 1.00 ± 0.00, n = 4, $p < 0.001$, paired sample t-test, Fig. 3A). VD supplementation rescued this effect as Group IV mice (HD +

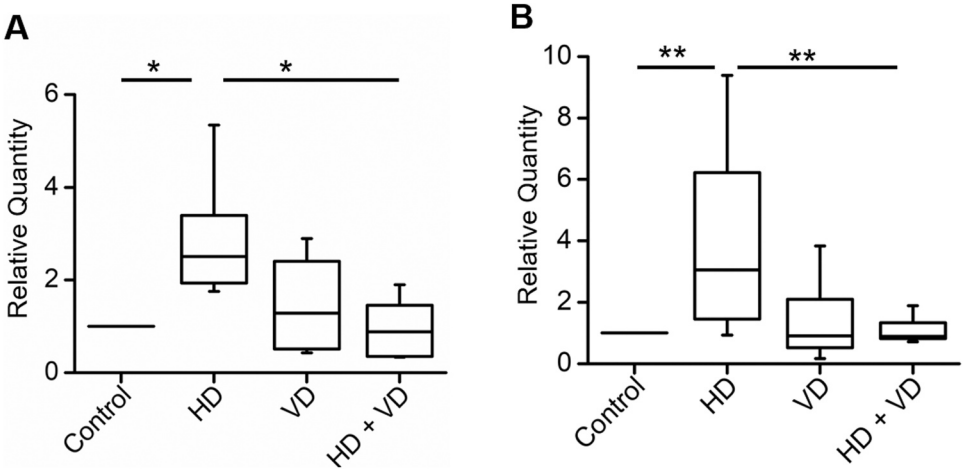

Fig. 2. Vitamin D3 (VD) intake decreases the gene expression of the TCR-β subunit in the cortex and striatum of HD mice. (A) Data demonstrating a significant increase in the cortical gene expression of the TCR-β subunit in Group II mice (HD vs control; n = 6, $p = 0.009$, paired sample t-test). VD administration to Group IV mice post-3-NP injection rescued the mRNA expression of the TCR-β subunit in the cortex of HD mice (HD + VD vs HD; n = 6, $p = 0.02$, paired sample t-test). (B) RT-PCR results depicting VD administration decreased the mRNA expression of the TCR-β subunit in the striatum of 3-NP induced HD mice (HD + VD vs HD; n = 10, $p = 0.008$, paired sample t-test). TCR-β subunit expression was significantly upregulated in Group II mice as compared to Group I mice (HD vs control; n = 10, $p = 0.005$, paired sample t-test). Data is represented as box-and-whisker plots depicting the median with first and third quartiles and whiskers representing the 5th and 95th percentile values.

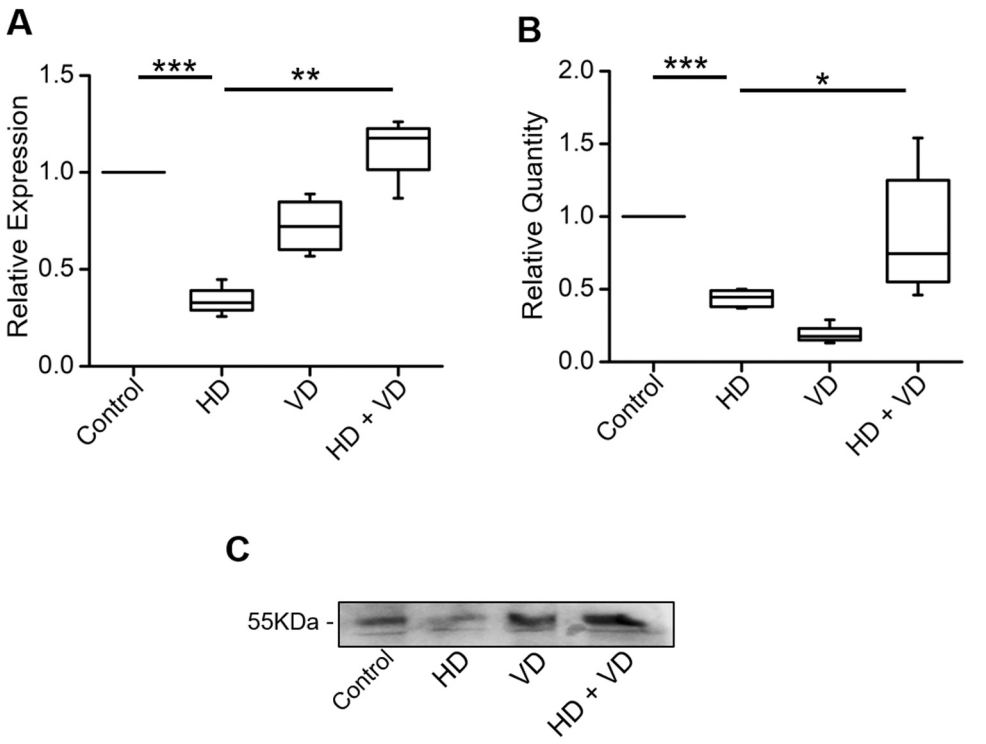

Fig. 3. Effect of VD supplementation on the protein and gene expression of α7 nicotinic acetylcholine receptors (α7 nAChRs) in the cortex and striatum of HD mice (A) On the 30th day, an overall change in the protein expression of α7 nAChRs was observed in cortical tissue samples from all the four groups of mice ($n = 4$, $p < 0.001$, one-way ANOVA). VD supplementation rescued the cortical expression of α7 nAChRs in Group IV mice (HD + VD) as compared to Group II (HD) mice ($n = 4$, $p < 0.001$, paired sample t-test). (B) A significant increase in the mRNA expression of α7 nAChRs was also observed in the striatal samples of 3-NP induced HD mice on VD administration (Group IV vs Group II, $n = 6$, $p = 0.02$, paired sample t-test). The mRNA expression of α7 nAChRs got significantly decreased in HD mice when compared with control mice (Group II vs Group I; $n = 6$, $p < 0.001$, paired sample t-test). Data is represented as box-and-whisker plots depicting the median in the first and third quartiles and whiskers represent the 5th and 95th percentile values. (C) Representative gel image for protein expression of α7 nAChRs from the cortical tissues.

VD) showed an enhancement in the protein expression of α7 nAChRs as compared to Group II (HD) mice (1.13 ± 0.07 vs 0.24 ± 0.08, $n = 4$; $p < 0.001$, paired sample t-test, Figs. 3A, and 3C). Real-time PCR analysis conducted on the striatal sample also showed a dramatic decrease in the mRNA expression of α7 nAChRs in HD mice which got rescued on VD administration (Group II vs Group IV 0.44 ± 0.01 vs 0.88 ± 0.10, $n = 6$, $p = 0.02$, paired sample t-test, Fig. 3B). α7 nAChRs mRNA expression got subsided in HD mice when compared to control mice (Group II vs Group I 0.44 ± 0.01 vs 1.00 ± 0.00, $n = 6$, $p < 0.001$, paired sample t-test, Fig. 3B). These results indicate that an increase in the gene expression of TCR-β (Fig. 2) was somehow causing a negative regulation of α7 nAChRs expression in HD and validates our previous finding where we showed that the entire octameric component of activated TCR downregulated the expression and function of the α7 nicotinic acetylcholine receptors (Komal et al., 2014). Here we show that neurotoxic conditions mimicked by 3-NP cause an increase in the gene expression of native immune proteins like TCR-β with concomitant downregulation in protein and mRNA expression of α7 nAChRs in the central nervous system.

3.3. Vitamin D administration alleviates acetylcholinesterase levels in the cortex and striatum of HD mice

To analyze the effect of Vitamin D3 (VD) on cholinergic neurotransmission, acetylcholinesterase (AChE) activity assay was performed on the cortical and striatal tissue samples from the respective four groups of mice. HD mice induced with 3-NP (75 mg/kg) showed a significant rise in the AChE activity when compared with control mice (Group II vs Group I, 748 ± 70 mU/mg vs 417 ± 26 mU/mg, $n = 6$, $p < 0.001$, paired sample t-test, Fig. 4A), indicating the detrimental effect of 3-NP on cholinergic neurotransmission in the cortex. However, Vitamin D3 administration attenuated the effect of 3-NP and decreased the cortical AChE activity in HD mice (Group IV vs Group II; 502 ± 33 mU/mg vs 748 ± 70 mU/mg, $n = 6$, $p = 0.002$; paired sample t-test, Fig. 4A). A similar increase in AChE activity was also observed in the striatum of HD mice (Group II vs Group I, 49 ± 4 mU/mg vs 29 ± 3 mU/mg, $n = 8$, $p < 0.001$, paired sample t-test, Fig. 4B). On the 30th day, VD administration significantly attenuated the AChE activity in 3-NP induced mice (HD + VD) when compared with HD mice (Group IV vs

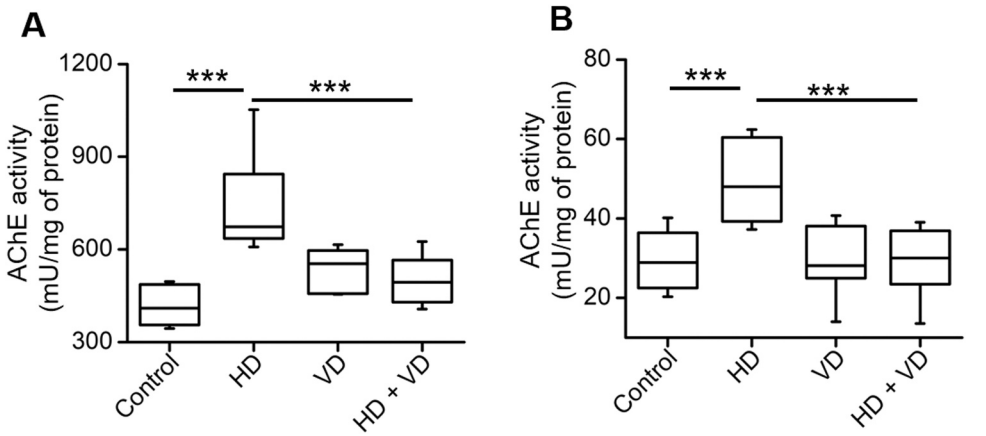

Fig. 4. Effect of VD supplementation on the enzymatic activity of acetylcholine esterase (AChE) in the cortex and striatum of HD mice (A) On the 30th day, a notable change in the activity of AChE was observed in the cortex of all four groups of mice ($n = 6$, $p < 0.001$, one-way ANOVA). VD induction significantly combated the activity of AChE in Group IV mice as compared to Group II mice (HD + VD vs HD; $n = 6$, $p = 0.002$, paired sample t-test). (B) A significant decrease in the activity of AChE was also observed in the striatal brain tissue samples of Group IV mice, supplemented with VD (HD + VD vs HD, $n = 8$, $p < 0.001$, paired sample t-test). Data is represented as box-and-whisker plots depicting the median with first and third quartiles and whiskers representing the 5th and 95th percentile values.

Group II, 29 ± 3 mU/mg vs 49 ± 4 mU/mg, n = 8, p < 0.001, paired sample t-test, Fig. 4B). These results indicate an anti-cholinesterase effect of VD in HD. The results of this study are in accordance with a previous finding where VD attenuated the AChE activity in the cerebral cortex of diabetic rats (Rodrigues et al., 2019). Thus, VD supplementation can rescue deficits in cholinergic neurotransmission by decreasing AChE activity and restoring acetylcholine (ACh) levels in HD.

3.4. Vitamin D3 administration in HD mice decreases oxidative stress as reflected by a reduction in key antioxidants gene marker expression in the cortex

To elucidate the effect of VD supplementation on the gene expressions of antioxidant markers, we performed RT-PCR in all four groups of mice (Group I to Group IV). mRNA expressions of glutathione peroxidase 4 (GpX4), and catalase (Cat) were subsequently analyzed in the cortical brain samples. On the 30th day after 3-NP-induction in HD mice, an overall change in the gene expression of GpX4 in the cortical tissues was observed (n = 6, p < 0.001, one-way ANOVA, Fig. 5A). RT-PCR results of GpX4 revealed that 3-NP treatment elevated the gene expression of GpX4 in the murine cortex of HD mice as compared with control mice (Group II vs Group I; 2.83 ± 0.08 vs 1.00 ± 0.00, n = 6, p < 0.001, paired sample t-test, Fig. 5A). mRNA expression of GpX4 in Group IV mice (HD + VD) got significantly decreased on VD administration as compared to the HD mice (Group IV vs Group II; 1.31 ± 0.14 vs 2.83 ± 0.08, n = 6, p < 0.001, paired sample t-test, Fig. 5A).

The effect of VD supplementation showed a remarkable change in the gene expression of catalase (Cat) among all four groups of mice in cortical samples (n = 8, p = 0.004, one-way ANOVA, Fig. 5B). Cat mRNA expression was elevated in HD mice when compared with control mice (Group II vs Group I; 2.74 ± 0.33 vs 1.00 ± 0.00, n = 8, p = 0.008, paired sample t-test, Fig. 5B). HD mice on VD supplementation for 15 days showed a significant reduction in the mRNA expression of catalases in the cortex when compared with HD mice injected with 3-NP (Group IV vs Group II; 1.29 ± 0.16 vs 2.74 ± 0.33, n = 8, p = 0.003, paired sample t-test, Fig. 5B). A similar anti-oxidant effect of VD is demonstrated in our previous finding where a reduction in the gene expression of GpX4 and Cat was observed in the striatum of HD mice (Manjari et al., 2022). Overall, our data indicate a protective effect of Vitamin D3 (VD) in HD and suggest its therapeutic potential in maintaining the cortical and striatal functions in Huntington's disease (HD).

3.5. An Anti-inflammatory effect of Vitamin D3 supplementation in HD mice

A significant enhancement in the levels of pro-inflammatory cytokines like tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6) is known to precede striatal neurodegeneration in HD (Chambon et al., 2023; Jia et al., 2022). To validate if 3-NP induction causes neuroinflammation in the striatum, we analyzed the gene expression of vital neuroinflammatory markers like nuclear factor-kappa B (NF-κB), proinflammatory cytokines like TNF-α and IL-6 from the striatal and cortical brain tissue samples from all the four groups of mice. HD mice injected with 3-NP showed a profound enhancement in the gene expression of NF-κB as compared to the control mice (Group II vs Group I; 7.42 ± 0.25 vs 1.00 ± 0.00, n = 4, p < 0.001, paired sample t-test, Fig. 6A). The mRNA levels of TNF-α (Group II vs Group I; 1.64 ± 0.06 vs 1.00 ± 0.00, n = 4, p = 0.005, paired sample t-test, Fig. 6B) and IL-6 in the striatum were also elevated on 3-NP induction (Group II vs Group I; 3.89 ± 0.50 vs 1.00 ± 0.00; n = 4, p = 0.02, paired sample t-test, Fig. 6C). Upon Vitamin D3 administration, the mRNA expression of NF-κB significantly subsided in HD mice (Group IV vs Group II; 0.57 ± 0.04 vs 7.42 ± 0.25, n = 4, p < 0.001, paired sample t-test, Fig. 6A). VD intake by HD mice also showed a profound decrease in the mRNA expression of TNF-α (Group IV vs Group II; 1.04 ± 0.07 vs 1.64 ± 0.06, n = 4, p = 0.02, paired sample t-test, Fig. 6B) and IL-6 (Group IV vs Group II; 1.08 ± 0.13 vs 3.89 ± 0.50, n = 4, p = 0.01, paired sample t-test, Fig. 6C), reflecting its anti-inflammatory action in the striatum.

A similar antagonistic effect of VD on inflammatory cytokines gene expression was observed in the cortex of HD mice. An increase in the cortical mRNA expression of TNF-α got substantially decreased in 3-NP induced HD mice treated with Vitamin D3 (Group II vs Group I; 1.50 ± 0.07 vs 1.00 ± 0.00, n = 4, p = 0.01; Group IV vs Group II; 0.77 ± 0.03 vs 1.50 ± 0.07, n = 4, p = 0.002, paired sample t-test, Fig. 6D). Similarly, VD supplementation significantly decreased the mRNA expression of IL-6 in HD mice (Group IV vs Group II; 1.08 ± 0.07 vs 1.73 ± 0.14, n = 4, p = 0.01, paired sample t-test, Fig. 6E). Altogether, our data validate previous findings where HD pathogenesis was found to be associated with an aberrant NF-κB pathway activation (Khoshnan et al., 2004; Soylu-Kucharz et al., 2022).

4. Discussion

The two primary pathological mechanisms commonly observed across all neurodegenerative diseases including Huntington's disease are increased oxidative stress and neuroinflammation (Cherubini et al., 2020; Maity et al., 2022; Pérez-Rodríguez et al., 2020). Evidence indicates under these neurotoxic conditions there is an enhancement in the gene expression of the brain resident immune protein, the major histocompatibility complex-I (MHC-I, Wang et al., 2021; Welberg, 2013). Several studies also demonstrate that "immune receptors" like major histocompatibility complexes type I (MHC-I), the cluster of

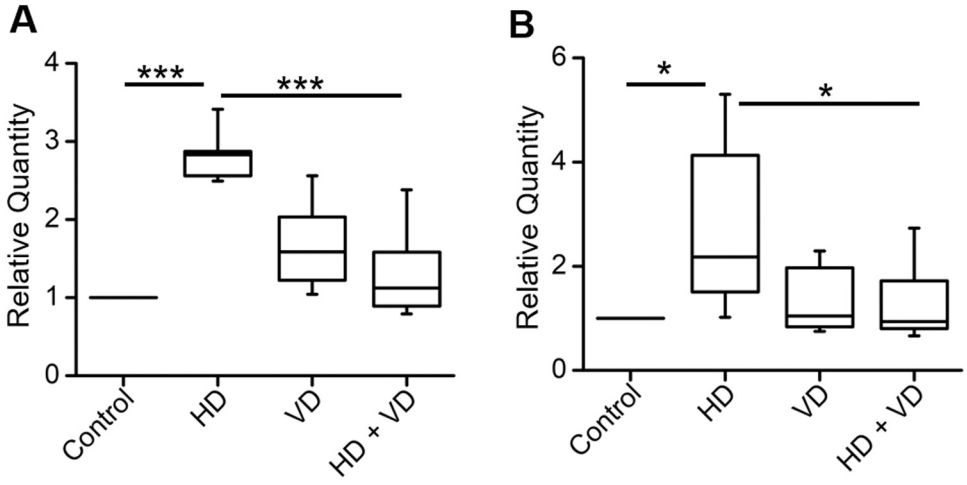

Fig. 5. VD administration rescues the gene expression of antioxidants in HD mice (A) mRNA expression of glutathione peroxidase 4 (GpX4) was increased in Group II mice (HD vs control, n = 6, p < 0.001, paired sample t-test), which subsided on VD supplementation (HD + VD vs HD, n = 6, p < 0.001, paired sample t-test) (B) The mRNA expression of catalase (Cat) was also found to be increased in the cortex of Group II mice as compared to Group I mice (HD vs control, n = 8, p = 0.008, paired sample t-test). The gene expression of Cat got alleviated on VD supplementation in Group IV mice reflecting its antioxidant effect (HD + VD vs HD, n = 8, p = 0.003, paired sample t-test). Data is represented as box-and-whisker plots depicting the median with first and third quartiles and whiskers representing the 5th and 95th percentile values.

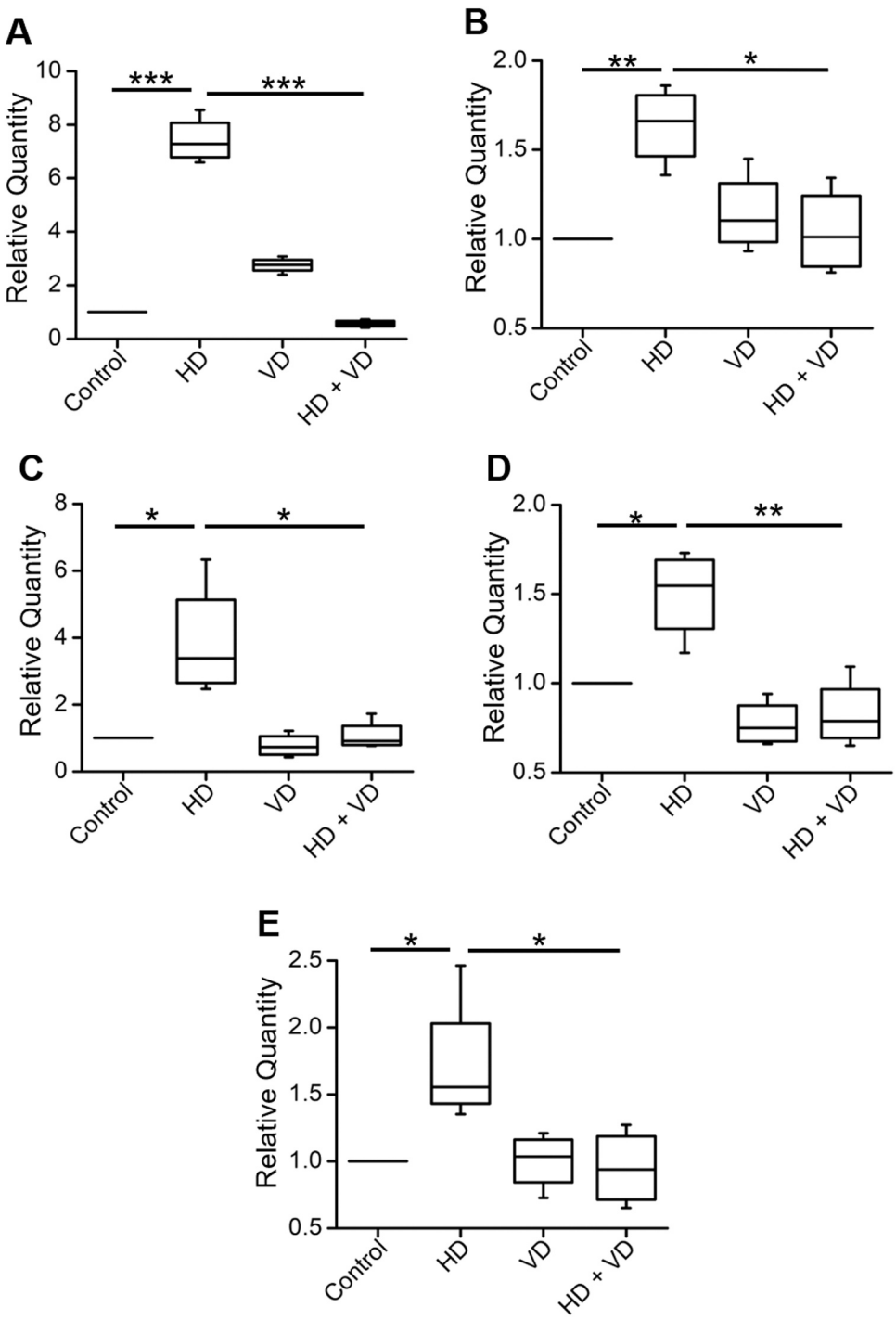

Fig. 6. An Anti-inflammatory effect of 500IU/kg of VD in the striatum and cortex of 3-NP induced HD mice. (A) mRNA expression of nuclear factor kappa B (NF-κB) was significantly increased in Group II mice (HD vs control, $n = 4$, $p < 0.001$, paired sample t-test) which got combated on VD supplementation (HD + VD vs HD, $n = 4$, $p < 0.001$, paired sample t-test). (B) Group IV mice administered with VD showed a significant reduction in the striatal gene expression of TNF-α (HD + VD vs HD, $n = 4$, $p = 0.02$, paired sample t-test) which got elevated in Group II mice injected with 75 mg/kg of 3-NP (HD vs control, $n = 4$, $p = 0.005$, paired sample t-test). (C) An increased mRNA expression of another inflammatory cytokine, interleukin 6 (IL-6) was observed in the striatum of Group II mice (HD vs control, $n = 4$, $p = 0.02$, paired sample t-test) which got subsided in Group IV mice when administered with VD (HD + VD vs HD, $n = 4$, $p = 0.01$, paired sample t-test). (D) A similar increase in the mRNA expression of tumor necrosis factor-α (TNF-α) was observed in the cortical brain tissue samples of Group II mice injected with 3-NP (HD vs control, $n = 4$, $p = 0.01$, paired sample t-test). The post-supplementation of VD for 15 days significantly attenuated the gene expression of TNF-α (HD + VD vs HD, $n = 4$, $p = 0.002$, paired sample t-test) and (E) interleukin 6 (IL-6) in the cortex of Group II mice (HD + VD vs HD, $n = 4$, $p = 0.01$, paired sample t-test). IL-6 gene expression was observed to be highly elevated in Group II mice on 3-NP injection (HD vs control, $n = 4$, $p = 0.03$, paired sample t-test). Data is represented as box-and-whisker plots depicting the median with first and third quartiles and whiskers representing 5th and 95th percentile values.

differentiation-zeta (CD-3ζ), and leukocyte immunoglobulin-like receptor B2 (LILRB2) play a key role in neurodegenerative disorders and could be a potential therapeutic target for neurological disorders like Alzheimer's disease (AD) and Parkinson's disease (PD) (Kim et al., 2013; Welberg, 2013). However, the brain's resident T-cell receptor beta subunit's (TCR-β) gene expression modulation in a neurological disorder like Huntington's disease (HD) is limited and remains largely unexplored. In this regard, studies have confirmed the neuroprotective capacity of Vitamin D3 (VD) in combating neuroinflammation, and oxidative stress, and restoring cholinergic signaling in different neurodegenerative disease models (Calvello et al., 2017; Koduah et al., 2017; Lima et al., 2018; Manjari et al., 2022). A study by Rodrigues and colleagues specifically showed that Vitamin D3 (VD) upregulated Vitamin D receptor expression, restored oxidative damage, and decreased acetylcholinesterase (AChE) activity in a rodent model of Alzheimer's disease (AD, Rodrigues et al.,2019). Our recent findings also highlighted the neuroprotective benefits of VD on motor dysfunction in 3-NP induced HD mice (Manjari et al., 2022).

In the present study, we demonstrate that a prolonged administration of 500IU/kg/day of Vitamin D3 (0–15 days) shows a long-lasting neuroprotective and anti-neurotoxic effect by decreasing the gene expression of the immune receptor, TCR-β subunit expression in both the cortex and striatum of HD mice (Fig. 2A and B). 3-NP administration is known to induce HD-like symptoms in rodents with a phenotype similar

to the genetically inherited human disease (Brouillet, 2014; Brouillet et al., 2005). The striatal medium spiny neurons are more susceptible to neurotoxic conditions induced by 3-NP as compared to the cortical neurons (Singh et al., 2010). A significant increase in inflammatory mediators such as tissue necrosis factor-alpha (TNF-α) is also reported previously to be associated with the neurodegenerative effects of 3-NP in the striatum (Ahuja et al., 2008). Under such a neurotoxic environment, here we show that there is an increased gene expression of native immune proteins in the T-cell receptor- beta (TCR-β) subunit with no change in the gene expression of the T-cell receptor-alpha (TCR-α) subunit in the HD mice (Supplementary fig.). We demonstrate that 3-NP induced increased oxidative stress causes a profound enhancement in the gene expression of TCR-β in the murine cortex and striatum which gets subsided on VD administration (Fig. 2A and B). We also found that 3-NP mediated enhancement in a free radical generation increased oxidative stress, and an increase in TCR-β subunit in HD mice was paralleled with an increase in acetylcholinesterase (AChE) activity in the two brain regions most vulnerable to undergo neuronal atrophy in HD i. e the cortex and the striatum (Fig. 4A and B).

AChE is an important regulatory enzyme found in cholinergic neurons and its elevation indirectly reflects cholinergic dysfunction (Walczak-Nowicka and Herbet, 2021). Cholinergic deficiency and an increase in AChE levels have been shown previously to cause memory impairment in the 3-NP induced rat model of HD (Menze et al., 2015). We show that Vitamin D3 (VD) administration decreases AChE activity in the cortex and the striatum which also possibly reflects its importance as therapeutics to combat neuronal loss observed in this neurodegenerative disease (Vattakatuchery and Kurien, 2013; Walczak-Nowicka and Herbet, 2021). Much of the therapeutic potential of VD is reflected in the studies performed on Alzheimer's disease (AD), where the neuroprotective mechanism occurred via Vitamin D receptor (VDR) signaling (Landel et al.,2016). In our recent finding, we also demonstrated that the striatal protein expression of VDR got rescued on post-VD supplementation in HD mice (Manjari et al., 2022). Hence, it is very likely that VD-VDR mediated upregulation of neurotrophins like brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) activates the neuronal survival pathway in HD (Manjari et al., 2022).

In the past, T-cell receptor (TCR) activation has been shown to negatively regulate the expression and function of α7 nicotinic acetylcholine receptors in the murine frontal and prefrontal cortex (Komal et al., 2014). Also, previous studies have demonstrated that the α7 cholinergic receptor's activity is modulated via a variety of kinases like Protein kinase A (PKA) and Src-family kinases like Lck and Fyn kinase (Komal et al., 2015, 2014; Komal and Nashmi, 2015). The α7 nicotinic acetylcholine receptor comes under the family of ligand-gated ion channels where these ionotropic receptors are known to contribute toward cognition, attention, and working memory function which gets compromised in neurological disorders (Dau et al., 2013; Komal et al., 2011; Perutz et al., 1999; Suzuki et al., 2006; Vattakatuchery and Kurien, 2013). It is possible that under a neuropathological insult like those observed in HD, which is characterized by elevated neuroinflammation, apoptotic signals, and oxidative stress, an enhanced gene expression of the TCR-β subunit occurs with a concomitant downregulation in the expression and function of alpha 7 nicotinic acetylcholine receptors (α7 nAChRs) potentiating neuronal loss in the striatum.

Vitamin D3 (VD) supplementation rescued the protein and mRNA expression of α7 nAChRs and also restored the acetylcholine levels with a simultaneous reduction in the immune receptor, TCR-β subunit mRNA expression in the cortex, and the striatal brain tissue samples (Fig. 2, Fig. 4 and Fig. 5). A restoration of cholinergic signaling in the striatum occurred with a downregulation in the gene expression of key proinflammatory cytokines like TNF-α and IL-6 in HD mice (Fig. 6). It is known that elevated levels of pro-inflammatory cytokines like TNF-α and NF-κB activity precede striatal neurodegeneration (Chambon et al., 2023; Khoshnan et al., 2004; Soylu-Kucharz et al., 2022). In our study, we show that VD intake by Group IV mice (HD + VD) showed a detrimental effect on NF-κB gene expression in the striatum (Fig. 6). Thus, the anti-inflammatory and anti-apoptotic effect of VD reflects its neuroprotective benefits as observed previously across a wide range of neurogenerative diseases including HD (Buell and Dawson-Hughes, 2008; Calvello et al., 2017; Chabas et al., 2008; Lima et al., 2018; Manjari et al., 2022; Mohamed et al., 2015; Nimitphong and Holick, 2011; Rodrigues et al., 2019).

VD mediates its biological effect by interacting with the Vitamin D receptor (VDR, Landel et al.,2016). It is very likely that VD-VDR interaction mediates an anti-apoptotic signal by inhibiting the NF-κB mediated activation of vital pro-inflammatory cytokines gene expression and rescuing the cholinergic signaling deficits by combating AChE activity with a restoration in the expression of α7 nAChRs in the cortex and the striatum. It may be argued that early intervention with VD can be proposed to have therapeutic benefits over a range of neurological disorders including HD possibly by downregulation of T-cell receptor-beta subunit expression (TCR-β) and inhibition of NF-κB mediated inflammatory cytokine pathway. The enhanced TCR-β subunit expression in the brain is justifiable in our findings as TCR-α subunit gene expression remained unchanged in all four groups of mice (supplementary fig.). However, we cannot rule out the possibility of invasion of peripheral T-lymphocytes invasion in our 3-NP mouse model of HD which also disrupts blood-brain barrier permeability (Kim et al., 2003). As recently proposed in our review, a functional anomaly of only the TCR-β subunit in neuropathological conditions is hypothesized in this work (Komal et al., 2022). It is speculated that striatal and cortical synapses may undergo enhanced synaptic pruning in HD via MHC-I and TCR-β interaction under increased oxidative stress, enhanced neuroinflammation, and mitochondrial dysfunction, which precedes the neurodegenerative processes observed across the plethora of neurodegenerative diseases (Komal et al., 2022).

5. Conclusion and perspectives

A purely hypothetical theory is projected here where we think TCR-β may either be weakly associated with CD3-complex or can exist as a TCR-β-β dimer that can act as a functional protein as demonstrated previously from in-vitro findings (Punt et al., 1991; Oh et al., 2019). This novel mechanism of downstream signaling cascade initiated by TCR-β in neurons may dictate the selective neurodegeneration of striatal and cortical neurons via downstream activation of kinase cascade and substantially abrogate the function and expression of nicotinic acetylcholine receptors under a neuropathological insult characterized by mitochondrial dysfunction, ER stress, elevation in oxidative stress, ATP depletion and increased cytokine storm as observed in HD and other neurological disorders (Komal et al., 2022). These statements merit additional research and future experiments will shed deeper insights into whether VD can interfere with the aberrant synaptic pruning preceding neurodegeneration in HD.

CRediT authorship contribution statement

MSKV, PR, and PK designed, conducted and analyzed all the experiments. MSKV, RKC, and SM wrote portions of the manuscript. MSKV, SMA, and PK revised all the experiments of the paper and statistical analysis. PK performed the final edit of the manuscript.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ibneur.2023.07.001.

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