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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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Interleukin-6 (IL-6)

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).

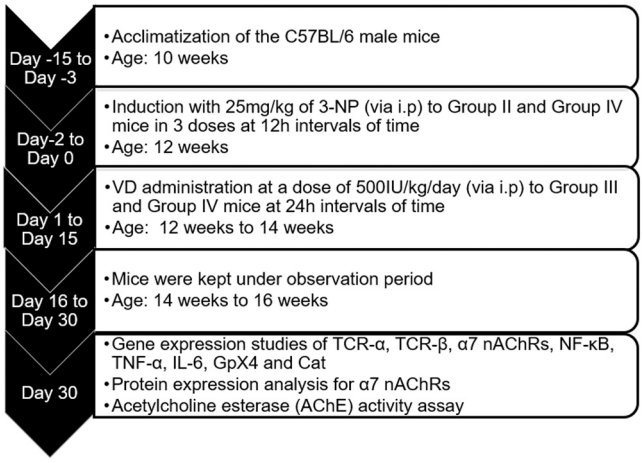


**Fig. 1.** Timeline and design for the study. C57BL/6 male mice at the age of ten to twelve weeks were undertaken in the present study. Mice were separated into four different groups. Group II and Group IV mice were injected (i.p) with 3-nitropropionic acid (3-NP) at 25 mg/kg dose at 12 h intervals 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 +

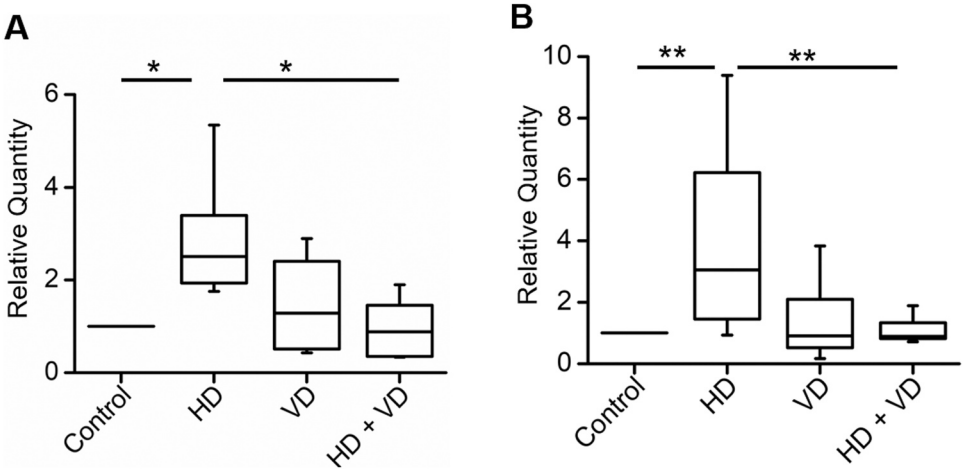


**Fig. 2.** Vitamin D3 (VD) intake decreases the gene expression of the TCR-β subunit in the cortex and striatum of HD mice. (A) Data demonstrating a significant increase in the cortical gene expression of the TCR-β subunit in Group II mice (HD vs control; n = 6, $p = 0.009$, paired sample t-test). VD 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.

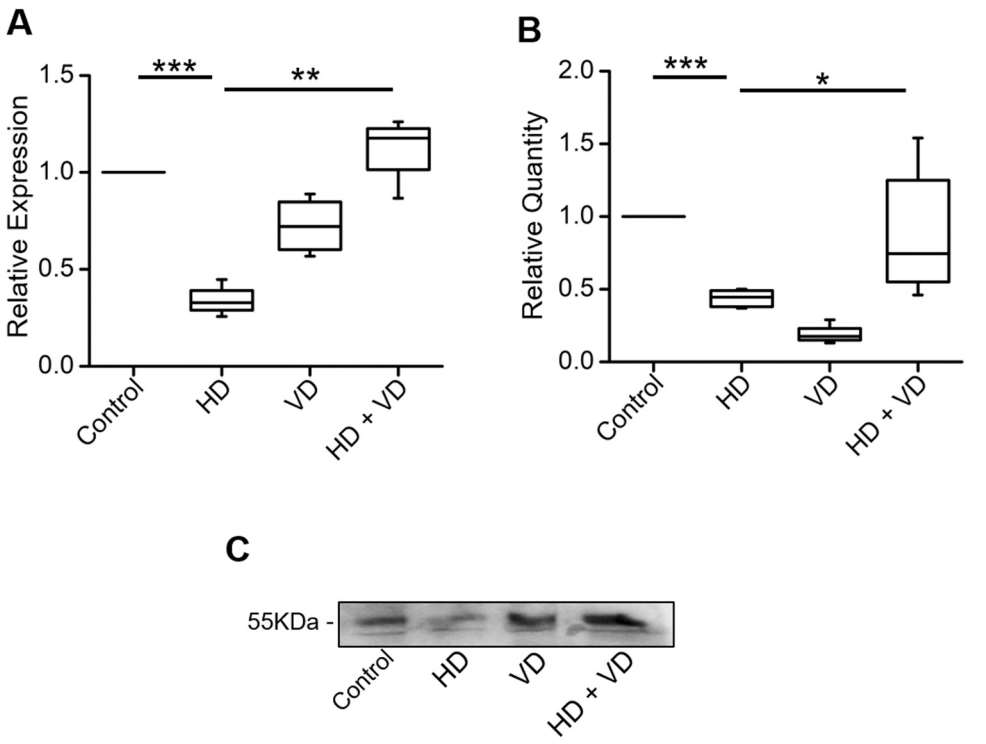


**Fig. 3.** Effect of VD supplementation on the protein and gene expression of α7 nicotinic acetylcholine receptors (α7 nAChRs) in the cortex and striatum of HD mice (A) On the 30th day, an overall change in the protein expression of α7 nAChRs was observed in cortical tissue samples from all the four 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 ($1.13 \pm 0.07$ vs $0.24 \pm 0.08$, $n = 4$; $p < 0.001$, 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 $0.44 \pm 0.01$ vs $0.88 \pm 0.10$, $n = 6$, $p = 0.02$, 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 $0.44 \pm 0.01$ vs $1.00 \pm 0.00$, $n = 6$, $p < 0.001$, 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, $748 \pm 70$ mU/mg vs $417 \pm 26$ 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; $502 \pm 33$ mU/mg vs $748 \pm 70$ 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, $49 \pm 4$ mU/mg vs $29 \pm 3$ 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

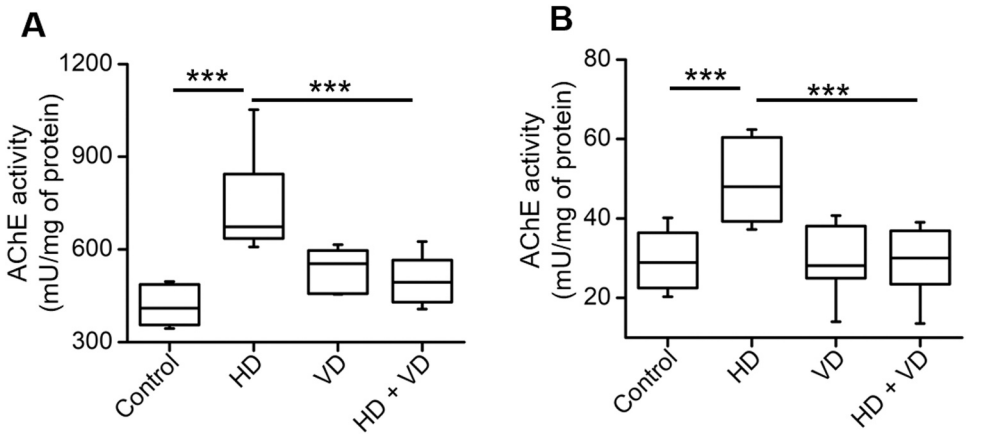


**Fig. 4.** Effect of VD supplementation on the enzymatic activity of acetylcholine esterase (AChE) in the cortex and striatum of HD mice (A) On the 30th day, a notable change in the activity of AChE was observed in the cortex of all four groups of mice ($n = 6$, $p < 0.001$, one-way ANOVA). VD induction 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

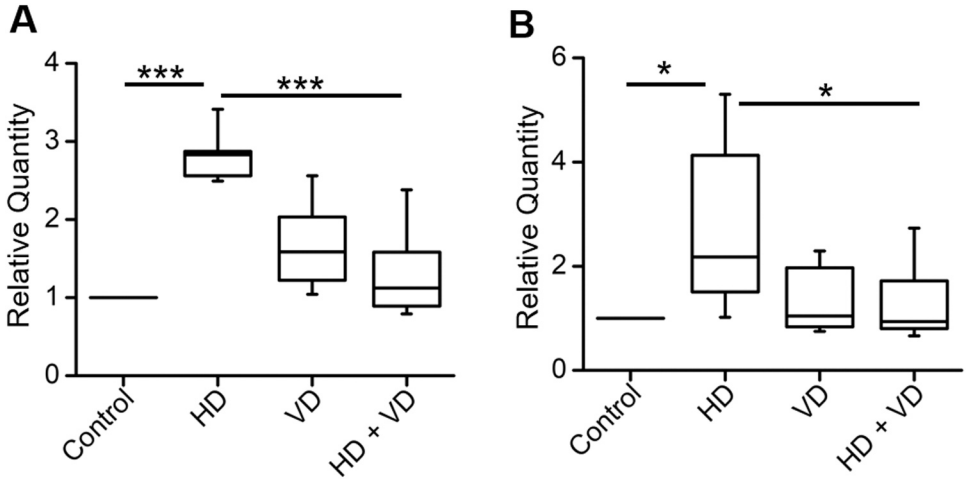


**Fig. 5.** VD administration rescues the gene expression of antioxidants in HD mice (A) mRNA expression of glutathione peroxidase 4 (GpX4) was increased in Group II mice (HD vs control, n = 6, p < 0.001, paired sample t-test), which subsided on VD supplementation (HD + VD vs HD, n = 6, p < 0.001, paired 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.

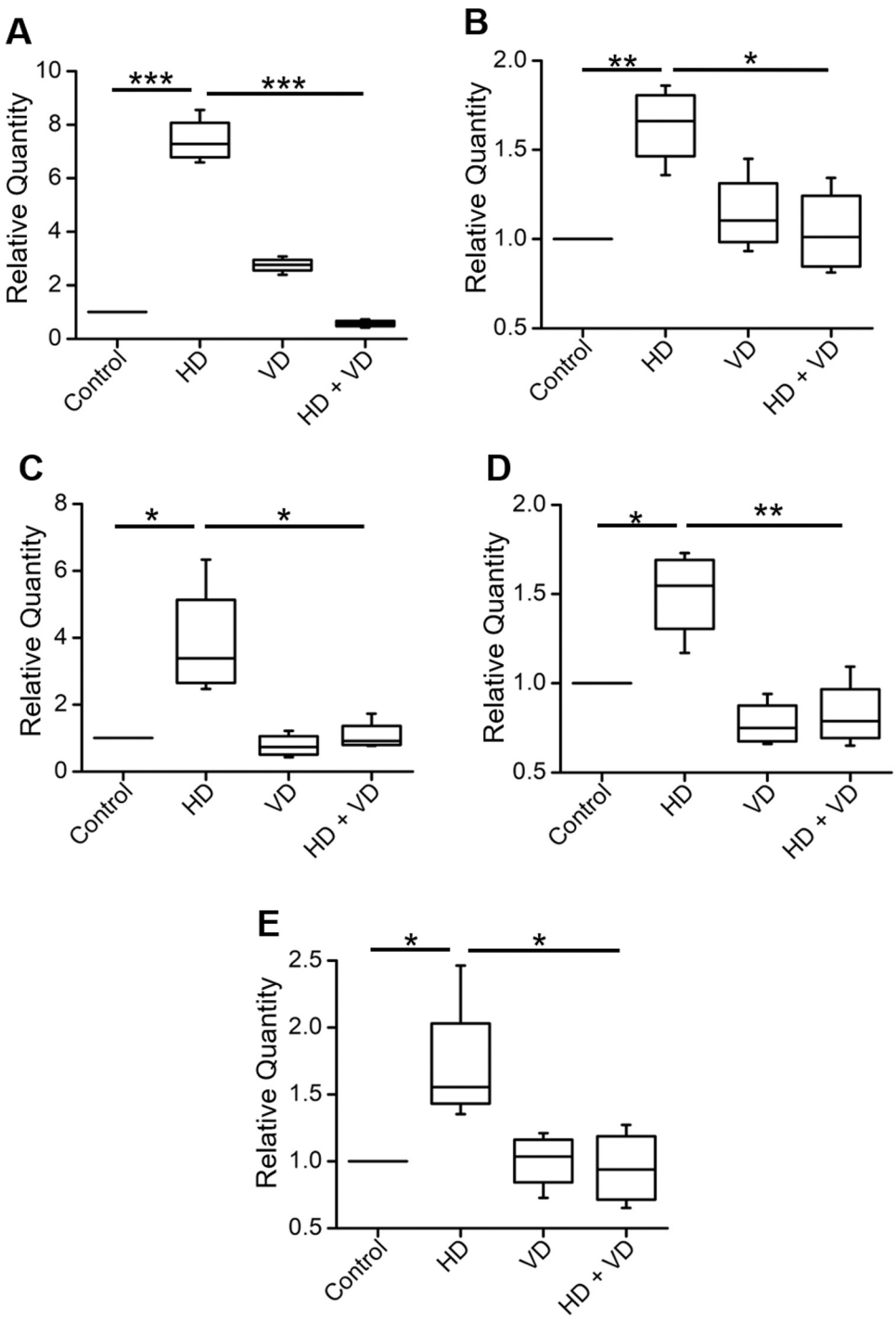


**Fig. 6.** An Anti-inflammatory effect of 500IU/kg of VD in the striatum and cortex of 3-NP induced HD mice. (A) mRNA expression of nuclear factor kappa B (NF-κB) was significantly increased in Group II mice (HD vs control, $n = 4$, $p < 0.001$, paired sample t-test) which got combated on VD supplementation (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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