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RESEARCH ARTICLE

# —NMDA R/+VDR pharmacological phenotype as a novel therapeutic target in relieving motor–cognitive impairments in Parkinsonism

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## Abstract

**Background:** Parkinsonism describes Parkinson's disease and other associated degenerative changes in the brain resulting in movement disorders. The motor cortex, extrapyramidal tracts and nigrostriatal tract are brain regions forming part of the motor neural system and are primary targets for drug or chemotoxins induced Parkinsonism. The cause of Parkinsonism has been described as wide and elusive, however, environmental toxins and drugs accounts for large percentage of spontaneous cases in humans. A common mechanism in the cause and progression of drug/chemotoxin induced Parkinsonism involves calcium signalling in; oxidative stress, autophagy, cytoskeletal instability and excitotoxicity

**Aim:** This study sets to investigate the effect of targeting calcium controlling receptors, specifically activation of Vitamin D<sub>3</sub> receptor (VDR) and inhibition of N-Methyl-D-Aspartate Receptor (NMDAR) in the motor cortex of mice model of drug induced Parkinsonism. Also we demonstrated how these interventions improved neural activity, cytoskeleton, glia/neuron count and motor–cognitive functions *in vivo*.

**Methods:** Adult mice were separated into six groups of  $n=5$  animals each. Body weight (5 mg/kg) of haloperidol was administered intraperitoneally for 7 days to block dopaminergic D<sub>2</sub> receptors and induce degeneration in the motor cortex following which an intervention of VDR agonist (VDRA), and (or) NMDAR inhibitor was administered for 7 days. A set of control animals received normal saline while a separate group of control animals received the combined intervention of VDRA and NMDAR inhibitor without prior treatment with haloperidol. Behavioral tests for motor and cognitive functions were carried out at the end of the treatment and intervention periods. Subsequently, neural activity in the motor cortex was recorded *in vivo* using unilateral wire electrodes. We also employed immunohistochemistry to demonstrate neuron, glia, neurofilament and proliferation in the motor cortex after haloperidol treatment and the intervention.

**Result/Discussion:** We observed a decline in motor function and memory index in the haloperidol treatment group when compared with the control. Similarly, there was a decline in neural activity in the motor cortex (a reduced depolarization peak frequency). General cell loss (neuron and glia) and depletion of neurofilament were characteristic anatomical changes seen in the motor cortex of this group. However, Vitamin D<sub>3</sub> intervention facilitated an improvement in motor–cognitive function, neural activity, glia/neuron survival and neurofilament expression. NMDAR inhibition and the combined intervention improved motor–cognitive functions but not as significant as values observed in VDRA intervention. Interestingly, animals treated with the combined intervention without prior haloperidol treatment showed a decline in motor function and neural activity.

**Conclusion:** Our findings suggest that calcium mediated toxicity is primary to the cause and progression of Parkinsonism and targeting receptors that primarily modulates calcium reduces the morphological and behavioral deficits in drug induced Parkinsonism. VDR activation was more effective than NMDAR inhibition and a combined intervention. We conclude that targeting VDR is key for controlling calcium toxicity in drug/chemotoxin induced Parkinsonism.

## Keywords

Calcium, cell death, degeneration, haldol, NMDAR, parkinsonism, VDR

## History

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## Introduction

Parkinsonism describes Parkinson's disease (PD) and (or) other associated movement disorders that can be observed in the behavior of an individual (Picillo et al., 2014; Zhu et al., 2014). Several discrete parts of the mammalian brain are known to be involved solely or in synergy with other brain areas in the cause and progression of Parkinsonism (Ricciardi et al., 2014). Although PD is the second leading cause of neurological disorders, the main cause has been described as wide and elusive. Some of the known features of Parkinsonism includes, degeneration of motor neurons, inflammation, depletion of dopamine in the nigrostriatal tract, hyper phosphorylation of cytoskeletal proteins and multifactorial genetic factors involved in spontaneous mutations in more than several genes (Konieczny et al., 2014; Liu et al., 2010; Sühs et al., 2014). Mutation of PD genes (Parkin and PINK 1), environmental influence on gene expression, mitochondria defects, drug and chemotoxins are some of the common cause of Parkinsonism already identified (Alcalay et al., 2014; Barbiero et al., 2014; Björklom et al., 2013; Liu et al., 2013; Osuntokun, 1981; Puri, 2014).

Anatomical changes in many areas of the brain have been described in Parkinsonism (Busceti et al., 2013); ultimately, selective vulnerability of neurons in the motor neural circuit is often implicated in prevalence of the disease (Biundo et al., 2013; Bosch-Bouju et al., 2013). It is known that cortical projections of the nigrostriatal tract synapse in the layers of the motor cortex which sends extra cortical fibers into the other brain areas to control motor function, thus, degeneration along this tract will impair motor coordination (Braak & Braak, 2000; Braak et al., 2000; Höglinger et al., 2003). The most significant change is often associated with loss of pyramidal cells in the motor area, extrapyramidal tracts and dopaminergic neurons of the nigrostriatal tract (Chou et al., 2014; Dehay et al., 2012; Engel et al., 2008; Patil et al., 2014). Factors that induces stress, inflammation or selectively targets these neurons are implicated as causative agents in Parkinsonism (Alcalay et al., 2014; Barbiero et al., 2014). For example, pigmented dopaminergic neurons of the nigrostriatal tracts are known to be selectively destroyed through direct administration of 6-OHDA into the medial fore brain bundle (mfb) or striatum leading to dopamine depletion (Walker et al., 2010). Also, prolonged pharmacological inhibition of dopaminergic D1/D2 receptors induces synaptic denervation in the nigrostriatal system (Faucheux et al., 2003; Plum et al., 2013), and inhibit striatal transmission to the motor cortex (Ferrer et al., 2012; Hou et al., 2010). In addition, heavy metals and chemotoxins generate reactive oxygen species (ROS) which induces calcium surge, membrane damage, cytoskeletal instability and cell death (Sugiyama & Sun, 2014). Despite the involvement of calcium toxicity in autophagy and cytoskeletal instability in cortical and striatal degeneration, dopamine replacement (L-DOPA) remains the most used treatment for PD. However, in therapeutic development, receptors involved in the control of calcium might be potential targets for relieving the synaptic denervation and associated degenerative changes in the brain.

Vitamin D3 receptor (VDRA) is a steroid receptor widely distributed in the brain and highly concentrated in the

pigmented nigrostriatal tract and motor cortex (Cui et al., 2013; Liu et al., 2013). It is suspected to be involved in melanin synthesis, cytoskeletal stability and the control of calcium in the motor area (Eyles et al., 2005). An indirect control of calcium in NMDAR inhibition has been described in reversal of excitotoxicity in *in vitro* and *in vivo* (Chen et al., 2013; Ibáñez-Sandoval et al., 2007). Furthermore, potentiation of the NMDAR is involved in glutamate excitotoxicity, degeneration in the nigrostriatal tract and motor cortex through the increase of inward calcium current that elevates cerebral calcium and induces excitotoxicity (Chen et al., 2013).

Interestingly, our previous findings show that activation of VDR by VDR agonists (VDRA) and inhibition of NMDAR *in vitro* reduces calcium toxicity and stabilizes microtubules, thus, preventing excitotoxicity and synaptic denervation (Blandini et al., 1996; Hurley & Dexter, 2012; Ogundele et al., 2014; Villalba & Smith, 2010). Despite the potential as therapeutic targets, VDR and NMDAR mediated calcium reduction in reversal of cortical degeneration (Parkinsonism) is still relatively unexplored. Using anatomical and electrophysiological approaches, we have elucidated the effect of VDR potentiation/NMDAR inhibition in improving cortical degenerative changes and neural activity of the motor cortex in drug-induced Parkinsonism. Furthermore we sought to identify, comparatively the effect of VDR potentiation and (or) NMDAR inhibition on neural depolarization potential and motor-cognitive functions *in vivo*.

## Methods

**Animals preparation:** Male adult BLAB/c mice weighing between 20 and 25 g were procured from the animal holding facility of Afe Babalola University. Animals ( $n=30$ ) were separated into three primary groups A, B and C. Group A ( $n=5$ ) received normal saline for 2 weeks; representing the negative control. Group B ( $n=15$ ) was treated with intraperitoneally administered aaloperidol (5 mg/kg BW) for 7 days, following which they were separated into three (3) sub-groups B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub> ( $n=5$  animals each). B<sub>1</sub> received Vitamin D<sub>3</sub> (VD<sub>3</sub>) (Oral; 70 mg/kg), B<sub>2</sub> was treated with 6 mg/kg BW of ketamine (intraperitoneal) while B<sub>3</sub> was treated with both VD<sub>3</sub> and ketamine for 7 days post-treatment with haloperidol. Group C received normal saline for 7 days following which they were treated with haloperidol (C<sub>1</sub>) and VD<sub>3</sub> + ketamine for 7 days (C<sub>2</sub>) ( $n=5$  each) (Table S2). All protocols were approved by the Animal Use Ethics Committee of the Afe Babalola University.

**Behavioral studies:** The animals were trained for 4 days in the behavioral testing area to allow them acclimatize to the room and the equipments to be used for each test to be conducted. Where necessary, scientists helped animals to move, navigate or climb to aid learning and preparation of the animals for the actual test.

**Rotarod test:** This was done to evaluate motor performance in the animals following treatment with haloperidol, ketamine (–NMDAR) and (or) vitamin D<sub>3</sub> (+VDR). Each test consists of three time trials on the rotarod (T<sub>1</sub>, T<sub>2</sub> and T<sub>3</sub>). It involves gradually increasing the speed from 3 rpms to reach a maximum speed of 35 rpm in 3 min for each trial ( $n=5$

animals in each group for each trial). An inter trial time (IT) of 20 min was observed for the three consecutive trials. A total of 15 trials was conducted for each group and was recorded using a high definition video recorder. Subsequently, we used inter reader variability to determine the maximum duration spent by an animal on the rotarod (latency of fall), also the number of passive rotations made in each trial.

**Open Field Test (OFT):** This was used to measure the exploration activity of the animals. A wooden cage open field area was marked to contain  $15 \times 15$  squares of  $18 \text{ cm} \times 18 \text{ cm}$  each. The distance travelled and the number of lines crossed were recorded using a high definition video recorder kept at a safe distance to cover the entire open field area (OFA). The frequency of line crossed was determined by using independent investigators to score the line crossing by each animal ( $n = 5$  per group).

**Elevated Plus Maze (EPM):** Using a modified design, anxiety linked behaviors were accessed in the animals ( $n = 5$  per group). These include the frequency of head dipping (HD), open arm duration (OAD), closed arm duration (CAD), closed arm entries (CAE) and open arm entries (OAE). The duration of the test was 3 min per mice.

**Novel Object Recognition (NOR):** Object recognition memory function was evaluated using the NOR test. A  $75 \text{ cm} \times 50 \text{ cm} \times 30 \text{ cm}$  transparent box was used. Three days prior to the habituation sessions, the mice were exposed to the box to familiarize with the environment. For the actual test, the animals were exposed to two identical objects for five minutes (Trial 1;  $T_1$ ). An inter trial time of 30 min was observed after which the animals were returned to the testing area for the second trial ( $T_2$ ). Time allowed for  $T_2$  was 5 min during which one of the old objects was replaced with a novel object. Exploration was scored when the nose of the animal is less than 2 cm from the object, while sitting on the object was excluded. Using inter reader variability, the videos were analyzed to estimate the time spent on rearing old object and new object in  $T_2$  to calculate the memory index [time spent on new object/total time spent on rearing both objects  $\times 100$ ] (Li et al., 2011).

**Electrophysiology:** Animals were anaesthetized using an isofluorane chamber. A dental drill was used to make holes on specific stereotaxic coordinates to pin point the motor cortex. The wired electrodes in carbon tubes were passed into the motor cortex approximately 4.68 mm anterior to the bregma and 2.5 mm deep (Figure 1h). Subsequently, the electrodes were fixed to the cranium using a rubber platform and orthopaedic glue. The dissected region of the scalp was cleaned using an antiseptic agent and animals were allowed to stay for 48 h to recover and move freely in the cage. In order to record the depolarization potential *in vivo*, the animals were transferred to modified cages and the electrodes were connected to a spiker box (Backyard Brains, Ann Arbor, MI). Neural activity was captured using the Audacity Software (version 2.0.5) on PC and analyzed in SigView (Version 2.6.1 by Signal Labs) to determine the maximum peak frequency and time epoch depolarization potential (Figure 1g and h).

### Immunohistochemistry

The animals were anaesthetized using iofluorane and decapitated. The whole brain was excised and perfused in

cold artificial cerebrospinal fluid [ACSF: 125 mM NaCl, 25 mM  $\text{NaHCO}_3$ , 3 mM KCl, 1.25 mM  $\text{NaH}_2\text{PO}_4$ , 1 mM  $\text{MgCl}_2$ , 2 mM  $\text{CaCl}_2$  and 25 mM glucose maintained at  $4^\circ\text{C}$ ] and transferred to formolcalcium for 14 h. The brains were processed routinely to obtain paraffin wax embedded tissue block. Thick sagittal sections ( $7 \mu\text{m}$ ) were cut using a microtome (Leica, Wetzlar, Germany). Sections were recovered and placed in urea and microwaved for antigen retrieval immunohistochemistry. Immunostaining with antibodies for antimice GFAP, NSE, NF and Ki-67 (Novocastra; Leica Biosystems, Wetzlar, Germany) were used to examine the protein expression in the motor cortex. A dilution of 1:200 (in PBS) was used for all primary antibodies. The color reaction was developed using avidin-biotin-peroxidase immunohistochemistry (Novocastra; Leica Biosystems, Wetzlar, Germany) and DAB (Sigma) as the polymer.

**Cell count and statistical analysis:** The images were acquired using an Optronics Digital Camera connected to a computer interface (MagnaFire) and an Olympus BX-51 Binocular research microscope (Olympus America, Newark, NJ). The cells were counted using Image J (NIH, Bethesda, MD) at a magnification of  $\times 100$ . For each image, the background was subtracted to remove noise following which the color threshold was set to generate black and white outline of cells in the section. Individual cells were denoted in binary mode of Image J (using the fill hole) followed by insertion of 1 pixel lines between cells (water shed; Image J). In subsequent processing, particle size was set at 128 pixels to eliminate smaller sized points and immunonegative cells in the sections. The count of immunopositive cells (GFAP, NSE, Ki-67 and NF) were determined at different microscopic fields ( $n = 7$ ) for  $n = 5$  sections in all groups. Data obtained was analyzed using ANOVA and Bon Ferroni Post Hoc test with significance set at  $p < 0.05$  [GraphPad Prism (Version 6.0)]. A  $p$  value of less than 0.05 was considered statistically significant, thus,  $p < 0.05$  (\*),  $p < 0.01$  (\*\*) and  $p < 0.001$  (\*\*\*).

**Note:** All behavioral test and cell count data were plotted as bar charts (mean) and error bars represents standard error of mean (SEM). Neural activity recording was presented as bar chart (mean of maximum frequencies) and standard deviation (error bar).

## Results

### Brain wave activities of the motor area

Comparing the peak activity and the root mean square (RMS) (extracellular neural recording with time epoch) for all groups, showed that the control recorded the highest neural activity (peak value of 32 440 Hz; Figure 1a and g) while the haloperidol treated group ( $\Delta\text{PD}$ ) had the lowest neural activity (429 Hz; Figure 1b and g). We probed further by comparing peak frequency for the control and  $\Delta\text{PD}$  with those of animals in which PD was induced followed by an intervention of NMDAR inhibition, VDR activation or both. A significant increase in neural activity was observed in the VDR activation group ( $\Delta\text{PD}/+\text{VDR}$ ; 32 364 Hz) and the combined intervention group ( $\Delta\text{PD}/+\text{VDR}/-\text{NMDAR}$ ; 32 442 Hz) when compared with the control (Figure 1d and f). Interestingly, we observed an increased peak in after blockade of the NMDAR ( $\Delta\text{PD}/-\text{NMDAR}$ ; 25 678 Hz) when



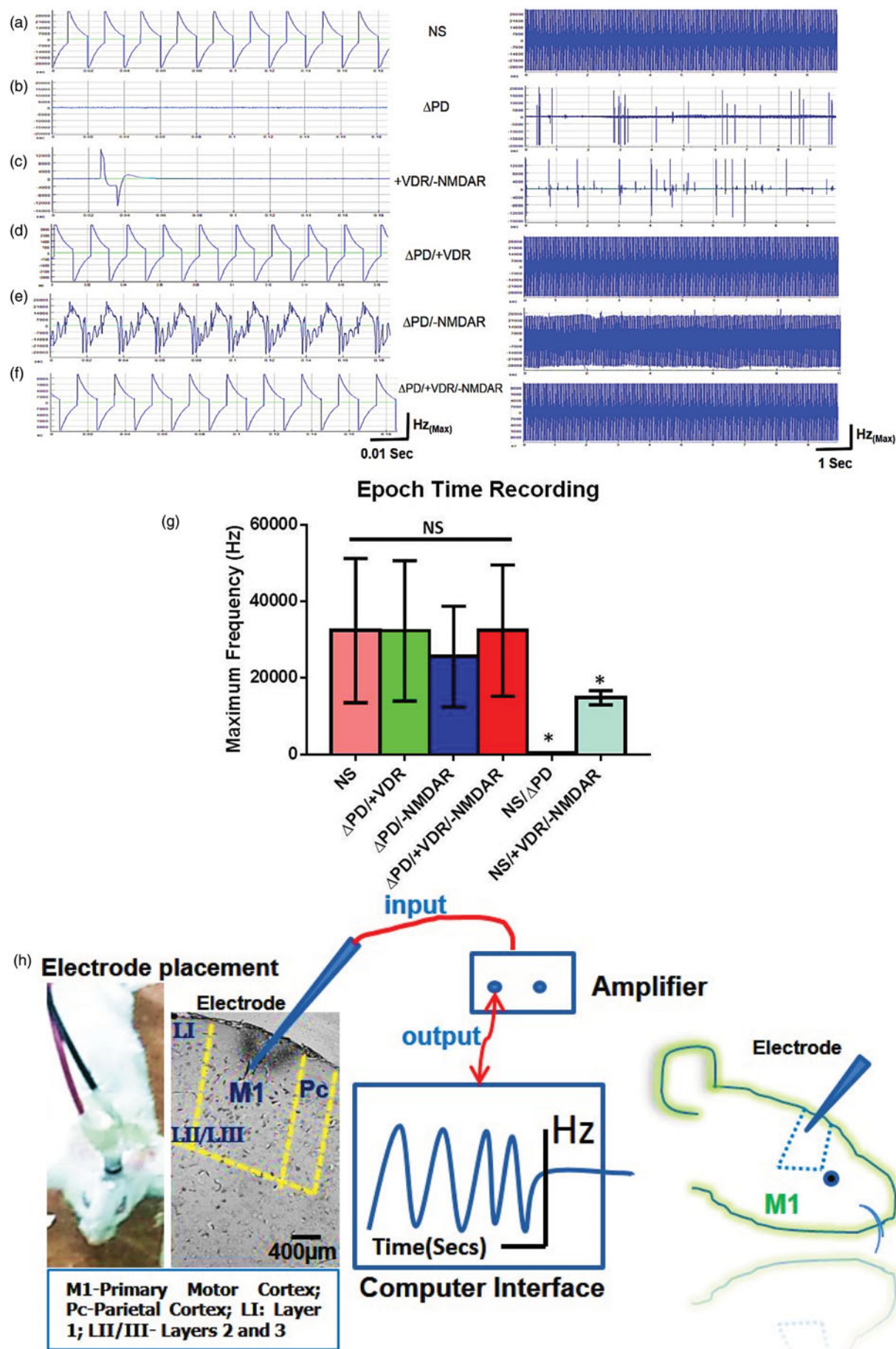


Figure 1. (a–f) Unilateral electrode recording from the motor area of mice. (a) Neural depolarization potential recording in the motor cortex of the control animals with a maximum peak 32 440 Hz and root mean square value (standard deviation) of  $7.165 \pm 0.09$ . (b) The lowest depolarization peak

Continued

compared to the  $\Delta$ PD and control, however, this was less significant when compared to the VDR activation or the combined intervention group (Figure 1e and e).

We next considered the amplified spikes (0.01 s) showing depolarization action potential (Figure 1g and Table S1) for the treatment groups and control. Because regulatory role of VDR activation and NMDAR inhibition is not well established, we analyzed depolarization phases in the motor cortex of the intervention groups to compare the neural activity for VDR activation versus NMDAR inhibition. NMDAR inhibition ( $\Delta$ PD/ $-$ NMDAR) caused repeated irregular depolarization spikes while the  $\Delta$ PD/ $+$ VDR and  $\Delta$ PD/ $+$ VDR/ $-$ NMDAR showed spike patterns similar to the control (although a time delay of 0.02 s was observed in these groups when compared with the control). The effect of the intervention was examined in a separate set up of control animals in which PD was not induced but animals received intervention for 7 days. A reduction in neural activity was observed ( $+$ VDR/ $-$ NMDAR; 14 870 Hz) when compared with the control and the PD intervention groups (Figure 1c and g). Subsequently, we compared the neural time epoch recording *in vivo* to the observed motor-cognitive function across the groups using behavioral assays. After prolonged pharmacological inhibition of dopaminergic (D2) receptor with haloperidol, the animals showed delayed motor response and staggered gait in motor function tests. Similarly, animals that received intervention without prior induced PD showed a reduced motor coordination (Figures 2 and, 3a and b).

### Motor function

In order to determine the significance of the observations in neural epoch recording after induced PD and intervention, the motor function test outcomes were compared with the cortical neural activity *in vivo* (Figure 1g). Motor function and coordination were measured using batteries of tests to assess exploratory movement (open field test) and forced motor coordination (Rotarod). To reduce the margin of error, four (4) blinded investigators analyzed the videos independently. Dopaminergic (D<sub>2</sub>) inhibition with haloperidol impaired exploratory motor function significantly when compared with the control ( $p < 0.001$ ) (Figure 2). Similar to our observations in cortical neural activity recordings, intervention through VDR activation ( $\Delta$ PD/ $+$ VDR) improved the exploratory activity ( $p < 0.05$ ) while NMDAR inhibition

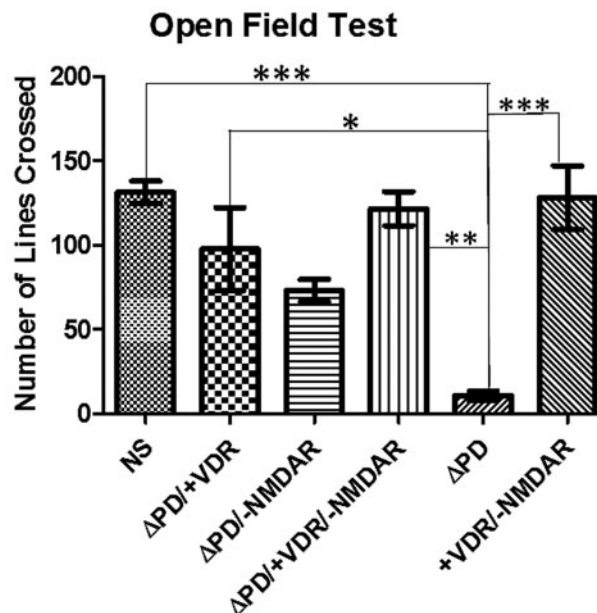


Figure 2. Frequency of lines crossed in OFT. Exploratory motor function was measured in OFT. The control recorded the highest frequency of lines crossed while the haloperidol treatment with no intervention ( $\Delta$ PD) had the least exploratory activity in this test. Similarly, VDR activation ( $\Delta$ PD/ $+$ VDR;  $p < 0.05$ ) and a combined treatment ( $\Delta$ PD/ $+$ VDR/ $-$ NMDAR;  $p < 0.01$ ) caused an improvement in exploratory motor function when compared to the haloperidol treatment group without intervention ( $\Delta$ PD). Pharmacological intervention through NMDAR blockade ( $\Delta$ PD/ $-$ NMDAR) gave no significant change in motor function when compared to the  $\Delta$ PD and control. Also, animals that received intervention without prior haloperidol treatment showed an increase in exploratory activity when compared to the  $\Delta$ PD ( $*p < 0.05$ ,  $**p < 0.01$  and  $***p < 0.001$ ).

( $\Delta$ PD/ $-$ NMDAR) caused no significant change when compared to the  $\Delta$ PD and control. In rotarod test, the latency of fall and number of passive rotations were quantified statistically. The  $\Delta$ PD recorded the least latency of fall (LOF), that is the least duration on the treadmill, while the control had the highest latency of fall ( $p < 0.05$ ) (Figure 3a). Motor coordination improved significantly in the VDR-activation intervention group when compared with the control and  $\Delta$ PD ( $p < 0.05$ ). However,  $\Delta$ PD/ $-$ NMDAR and  $\Delta$ PD/ $+$ VDR/ $-$ NMDAR showed no significant change in the LOF when compared with the control but was significant versus the  $\Delta$ PD (Figure 3a). The least frequency of passive rotations was

### Figure caption Continued

was observed in the  $\Delta$ PD (429 Hz) after the administration of haloperidol. Motor-cognitive impairment was also observed in this group. (c) A separate treatment group in which VDR and NMDAR antagonists were administered without any prior haloperidol treatment ( $NS/+VDR/-NMDAR$ ) showed a reduction in neural activity and decline in motor function. A maximum depolarization peak of 14 870 Hz was recorded in this group. This is probably due to the effect of this treatment in reducing cerebral calcium to a level below normal in untreated animals. (d) An improved neural activity was recorded in the VDR activation intervention group after initial haloperidol treatment and impaired motor function ( $\Delta$ PD/ $+$ VDR). A maximum peak value of 32 364 Hz was recorded for this group with a RMS value of  $6.96 \pm 0.09$  similar to the value obtained in the control. (e) An irregular depolarization potential was observed in the haloperidol treatment mice after an intervention with NMDAR antagonist *in vivo*.  $\Delta$ PD/ $-$ NMDAR recorded a maximum peak of 25 678 Hz significantly lower than that of the control and VDR activation intervention groups. (f) A separate group of animals treated with haloperidol received a combined intervention of NMDAR blockade and VDR activation ( $\Delta$ PD/ $+$ VDR/ $-$ NMDAR). Neural activity improved after the treatment with maximum peak reaching 32 442 Hz, similar to the control and the  $\Delta$ PD/ $+$ VDR. Animals in this category also showed an improved motor function in behavioral test. (g-h) Statistical analysis of neural activity recording. (g) Unilateral electrodes were placed in the left motor cortex while the animals engage in motor gestures in a restricted area. The maximum frequency (bar chart) denotes the peak depolarization potential recorded while the extracellular neural activity epoch (root mean square) is expressed as the standard deviation for each group (error bar). A decline in neural activity in time epoch recording was observed in the haloperidol treatment group and significantly increased in all the intervention groups ( $p < 0.05$ ). Neural activity also decreased in the intervention group without prior haloperidol treatment ( $NS/+VDR/-NMDAR$ ). (h) Electrode placement in the motor cortex of adult mice. The wire electrodes were placed in the layer 2/3 which receives somatosensory thalamocortical and striatal afferents responsible for motor coordination in the neural circuit of the motor cortex ( $*p < 0.05$ ).

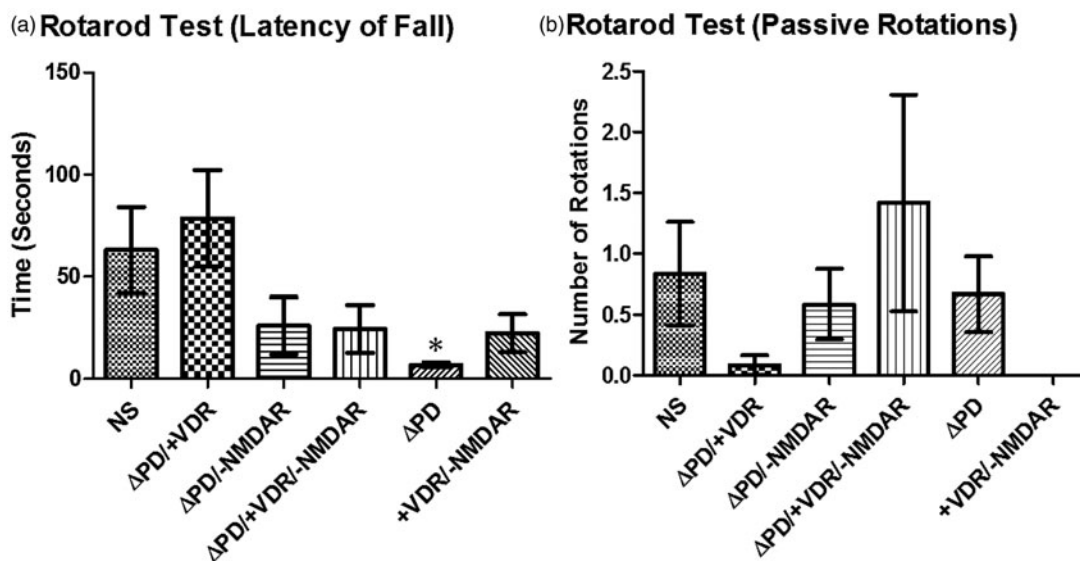


Figure 3. Rotarod test for motor function expressed as latency of fall (LOF) and number of passive rotations (PRs). (a) The LOF was determined by estimating the duration spent by each animal on the rotarod when the speed was gradually increased from 4 to 34 rpm. The maximum time allowed was 3 min for each test. A total of  $n=5$  animals were used for the actual test of three trials T1, T2 and T3. After haloperidol treatment, ΔPD showed a decline in motor function ( $p<0.05$ ) when compared with the control. Haloperidol treated animals that received VDRA intervention (ΔPD/+VDR) recorded the longest duration on the rotarod supporting an improved motor activity initially demonstrated in OFT (Figure 2). Although the ΔPD/+VDR/-NMDAR showed an improved exploratory activity in OFT, a reduced motor function was recorded in rotarod test (similar to the ΔPD/-NMDAR) when compared with the control. However, all treatments and control gave scores higher than the ΔPD (significant at  $p<0.05^*$ ). (b) The number of passive rotations (PRs) was recorded per animal for the three trials. The highest number of PRs was observed in the ΔPD/+VDR/-NMDAR which recorded a low LOF for the same test duration. The +VDR/-NMDAR recorded no PR while the ΔPD and ΔPD/-NMDAR recorded value close to the control. It is important to note that the ΔPD/+VDR recorded the highest latency of fall in (A) and the least frequency of passive rotations ( $*p<0.05$ ).

recorded in the VDRA intervention group when compared to the ΔPD while the group that received intervention without prior treatment (NS/+VDR/-NMDAR) recorded displayed passive rotations throughout the duration of the test (out layer).

### Non-motor function

Novel object recognition (NOR) test was administered to measure the spatial memory function. We estimated the average exploration time on old and new objects (Figure 4) and subsequently determined the memory index (MI). The control group recorded the highest exploration time on both objects while the ΔPD explored the old object more than the new (low memory index). After VDRA intervention, the ΔPD/+VDR showed an improved memory index when compared with the control ( $p<0.05$ ) and ΔPD ( $p<0.05$ ). Interestingly, the +VDR/-NMDAR did not explore the new object at all thus demonstrating a reduced memory index (Figure 4B) similar to the reduced motor function (Figures 2 and 3b) and neural activity (Figure 1g). Anxiety was evaluated in elevated plus maze (EPM) which included the open arm duration (OAD), closed arm duration (CAD), open arm entries (OAE), closed arm entries (CAE) and frequency of head dipping (HD). The ΔPD recorded the highest CAD duration, thus signifying anxiety (Figure 5a). The +VDR/-NMDAR recorded the highest OAD when compared with the other groups (Figure 5b). The number of OAE and CAE was highest in the ΔPD/+VDR while HD was least in this intervention group. This represents a reduced anxiety and improved exploratory behavior after VDRA

intervention when compared with the control and ΔPD (Figure 5c and d).

### Immunohistochemistry

**GFAP:** Glia activation and increased cell number were observed in the treatment groups. All treatments and control recorded glia scores (GFAP+) that was significantly higher than the ΔPD group ( $p<0.001$ ). Glia loss in the ΔPD treatment also correlated with general cell loss in the cortex of these animals (Figure 6a and d).

**NSE:** The expression level of the neuronal marker was higher in the ΔPD/+VDR treatment when compared with the control and the ΔPD treatment ( $p<0.001$ ). However, no significant difference was observed in the expression of NSE in the ΔPD, ΔPD/-NMDAR and +VDR/-NMDAR (Figure 6b and e).

**NF:** The ΔPD showed a significant decline in NF deposition within the motor cortex. This represents a loss of neuronal cytoskeleton and synaptic connections in the motor cortex. An increase in NF expression was observed in the intervention group after VDRA treatment (ΔPD/+VDR) with a significance of  $p<0.001$  when compared with the control and ΔPD. NF deposition also increased in the ΔPD/-NMDAR and the ΔPD/+VDR/-NMDA intervention groups (Figure 6c and f).

**Ki-67:** The ΔPD showed a decline in cell number which was observed both in the neuron (NSE) and glia (GFAP) count. The Ki-67(+) cell distribution increased in the VDRA intervention group (ΔPD/+VDR); this corresponded to the increase in GFAP(+) and NSE(+) cell count in the motor



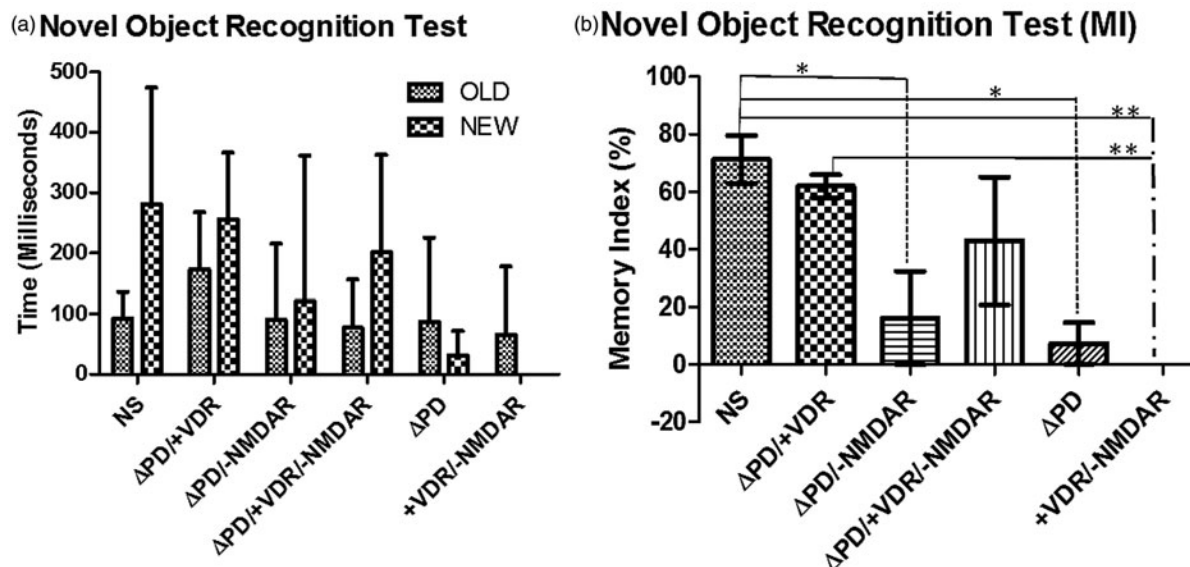


Figure 4. Novel object recognition test for spatial memory and memory index (MI). (a) Comparing the estimated time spent on new objects versus old objects, the control recorded the highest memory index while the haloperidol treatment had the lowest memory index. In addition, the control spent more time exploring both objects than all other groups, while the ΔPD recorded the least exploration time. An improved cognition was observed after the VDRA intervention in haloperidol treated mice (ΔPD/+VDRA) when compared with the control and the ΔPD ( $p < 0.01$ ). Similarly, cognitive function and memory consolidation increased in the haloperidol treatment group that received both interventions (ΔPD/+VDR/-NMDAR) as the animals explored the novel object and recorded an increase in memory index. (b) Bar chart showing the memory index for the treatment groups and control. A decline in memory index was seen in ΔPD and the intervention group without prior haloperidol treatment (NS/+VDR/-NMDAR). VDRA intervention in haloperidol treated mice (ΔPD/+VDR) caused an increase in the memory index when compared with the haloperidol treatment (ΔPD) without intervention ( $p < 0.001$ ). Also, the group that received intervention without prior haloperidol treatment showed a decline in memory index (NS/+VDR/-NMDAR). NMDAR inhibition (ΔPD/-NMDAR) and the combined intervention (ΔPD/+VDR/-NMDAR) caused no significant increase in memory index when compared to the ΔPD (\* $p < 0.05$ , \*\* $p < 0.01$ ).

cortex of this group. Ultimately, this indicates the role of VDRA treatment in improving cortical neuronal survival. Increased count and proliferation were also observed in the ΔPD/+VDR/-NMDAR ( $p < 0.01$ ) and ΔPD/-NMDAR ( $p < 0.05$ ), although not as significant as that in the ΔPD/+VDR ( $p < 0.001$ ) (Figure 7a and b).

## Discussion

Taken together, the findings of this study suggest that NMDAR and VDR receptor targeted mechanisms are involved in the reduction of intracellular calcium and are important in relieving cortical cytotoxicity and motor-cognitive impairments in Parkinsonism. Morphological evidence (Immunohistochemistry) showed that haloperidol induced Parkinsonism involves neuronal degeneration, reduced glia count and depletion of cortical neurofilament. This also translates into reduced neural activity in the motor cortex and such animals exhibited impaired motor and cognitive functions in behavioral studies. We have also shown that VDR potentiation and NMDAR inhibition reduce the cortical toxicity by improving neuronal metabolism (NSE), glia count (GFAP) and cytoskeleton to support synaptic function (NF), thus, an increase in neural activity and motor-cognitive function.

In this study haloperidol was selected as an inducing agent as its effect is encompassing. It can selectively target the cells of the pyramidal and extrapyramidal tract (Deepak et al., 2008; Espinoza et al., 2011) and inhibit dopaminergic receptors ( $D_2$ ) (Bertran-Gonzalez et al., 2009; Reavill et al., 1999). At cellular level, it induces synaptic denervation

through calcium-dependent depolymerization of microtubules in motor neurons (Cazorla et al., 2014). Furthermore, it increases glia and cellular activation, creating series of events similar to inflammation (Bishnoi et al., 2008; Voronkov et al., 2013). The symptoms of haloperidol induced movement disorders can result from the ability of the dopaminergic ( $D_2$ ) antagonist to induce oxidative stress and cause production of ROS (Byron et al., 2010). The ROS generally increase the calcium ion concentration by mobilizing calcium from intracellular and extracellular sources (Drago et al., 2013; Zhang et al., 2012). Such calcium accumulation leads to microtubule collapse and excessive phosphorylation of Tau, a microtubule associated protein involved in stabilizing the structure of the MT assembly in neurites (axon and dendrite) (Delotterie et al., 2010; Hasbi et al., 2009). Several studies have established the effect of Haloperidol in inducing cytoskeletal disorganization through ROS generation and hyper phosphorylation of Tau; in certain experiments 100 μM of Haloperidol, *in vitro*, caused a complete cytoskeletal collapse in most cells (Benítez-King et al., 2010a,b).

Previous studies have described the interplay between calcium toxicity, autophagy and synaptic denervation in Parkinsonism (Chen et al., 2013; Ibáñez-Sandoval et al., 2007; Villalba & Smith, 2010). Cellular activation of calcium through NMDAR and VDR are important in neuronal development, polarity, migration and synapse formation in the developing motor cortex. Similarly, these receptors are implicated in the pathology of degenerative diseases in the adult nervous system. Elevated cerebral calcium and glutamate coupled with a low serum level of Vitamin  $D_3$  is often



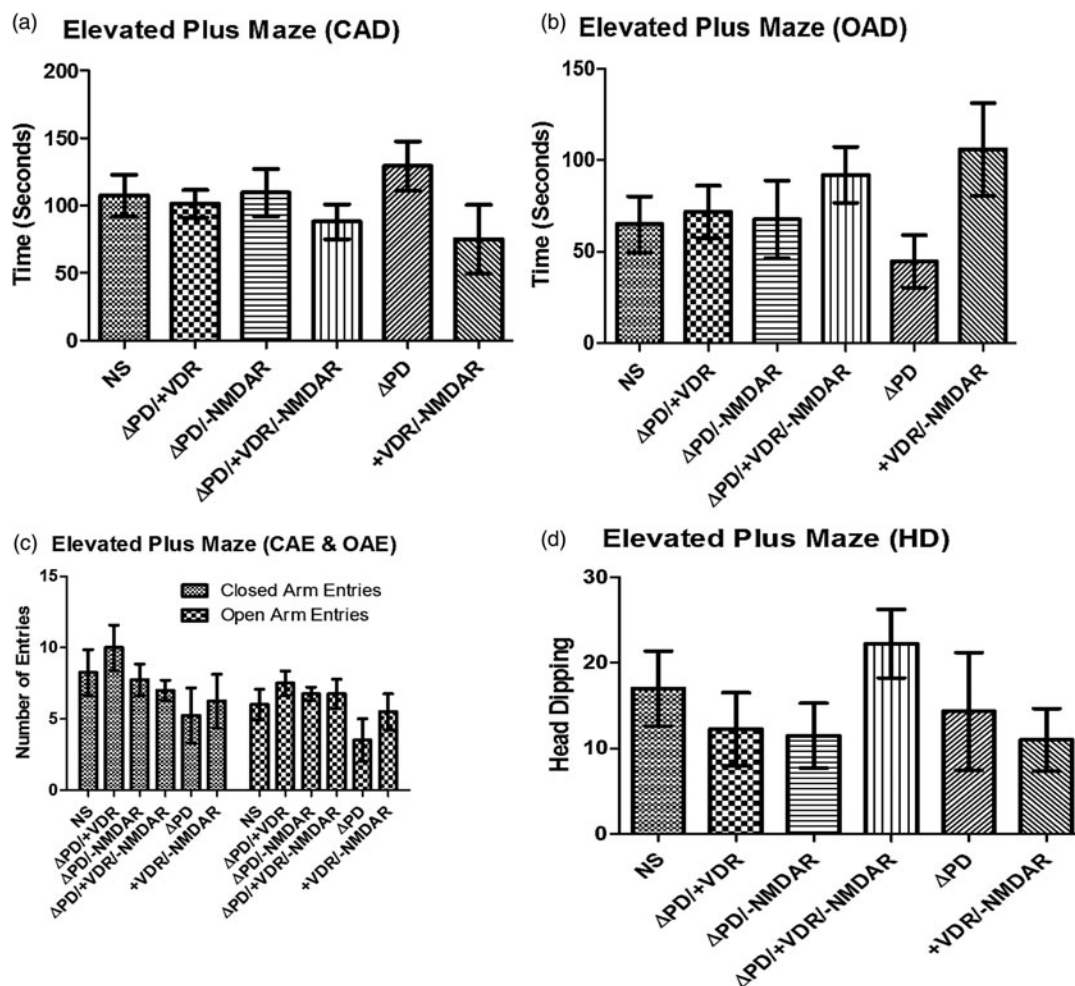


Figure 5. Elevated plus maze; a test for anxiety. (a) Closed arm duration (CAD) was highest in the  $\Delta PD$  thus signifying anxiety in this group, although the difference in CAD across the treatment and control was statistically insignificant. (b) OAD was highest in the  $+VDR/-NMDAR$  when compared to other groups. In the OFT this group also recorded a high exploratory motor function and reduced anxiety. (c) Composite bar chart comparing the number of CAE versus OAE. The  $\Delta PD/+VDR$  recorded the highest CAE and OAE while  $\Delta PD$  recorded the lowest in both. (d) The frequency of head dipping (HD) was plotted for each group to generate an average.  $\Delta PD/+VDR/-NMDAR$  showed the highest rate of HD. No significant difference was recorded in the anxiety-linked behaviors.

associated with Parkinsonism and other degenerative diseases (Peterson, 2014; Petersen et al., 2013, 2014).

Vitamin D<sub>3</sub> (VD) is important in neurodevelopment, up-regulation of neurotrophic factors, stabilization of mitochondrial function and antioxidation (Fuglestad et al., 2013; Harms et al., 2011; Orme et al., 2013). The VDR gene codes for the VDR is responsible for calcium regulation, immune response, neuronal functions (Castellano et al., 1998; Smolders et al., 2011). Improved motor and cognitive functions have been observed in amyotrophic lateral sclerosis (ALS) (Camu et al., 2014), Alzheimer's disease (Lu'o'ng & Nguyễn, 2011), PD (Peterson et al., 2013) and stroke patients (Makariou et al., 2014) receiving VDRA placebo supplements. Nissou et al. (2013) demonstrated that the role of VD goes beyond cellular mechanisms, over time it has been found to be associated with up regulation of several genes up to 1.9 folds at transcriptome level (Nakahashi et al., 2014; Wöbke et al., 2013). In addition, VDRA are known to influence dopaminergic neuron maturation, differentiation and neuronal melanogenesis through calcium dependent pathways activated by specific ligand receptors, such as endothelin receptor (Camu et al., 2014).

Although VDRs are highly localized in the brain, they are most predominant in the pigmented cell population of nigrostriatal tract; specifically their projections to the motor cortex and basal ganglia (Cui et al., 2013; Eyles et al., 2005). The role of VDR activation in reducing calcium ion concentration is important for stabilizing neuronal cytoskeletal structures, vesicle clearance and synaptic activities, thus, its activation (VDR+) reduces synaptic denervation and loss of dendritic spines in the motor pathway (Agholme et al., 2010; Clelland et al., 2014). Our results show that VDRA treatment ( $+VDR$ ) was more effective in improving cortical structure and function following haloperidol induced cell loss in the motor cortex. Neuronal and glia cell count increased in the VDRA intervention group and was significant when compared to NMDAR inhibition and the combined intervention (Figure 6a and d, b and e). VDRA intervention was also associated with an increase in NF deposition in the motor cortex (Figure 6c and f), increased neural activity (Figure 1) and motor function (Figures 2 and 3). The significant improvement observed after VDRA intervention can be attributed to the role of VDRA in mobilization of calcium for sequestration, thus reducing the intracellular calcium

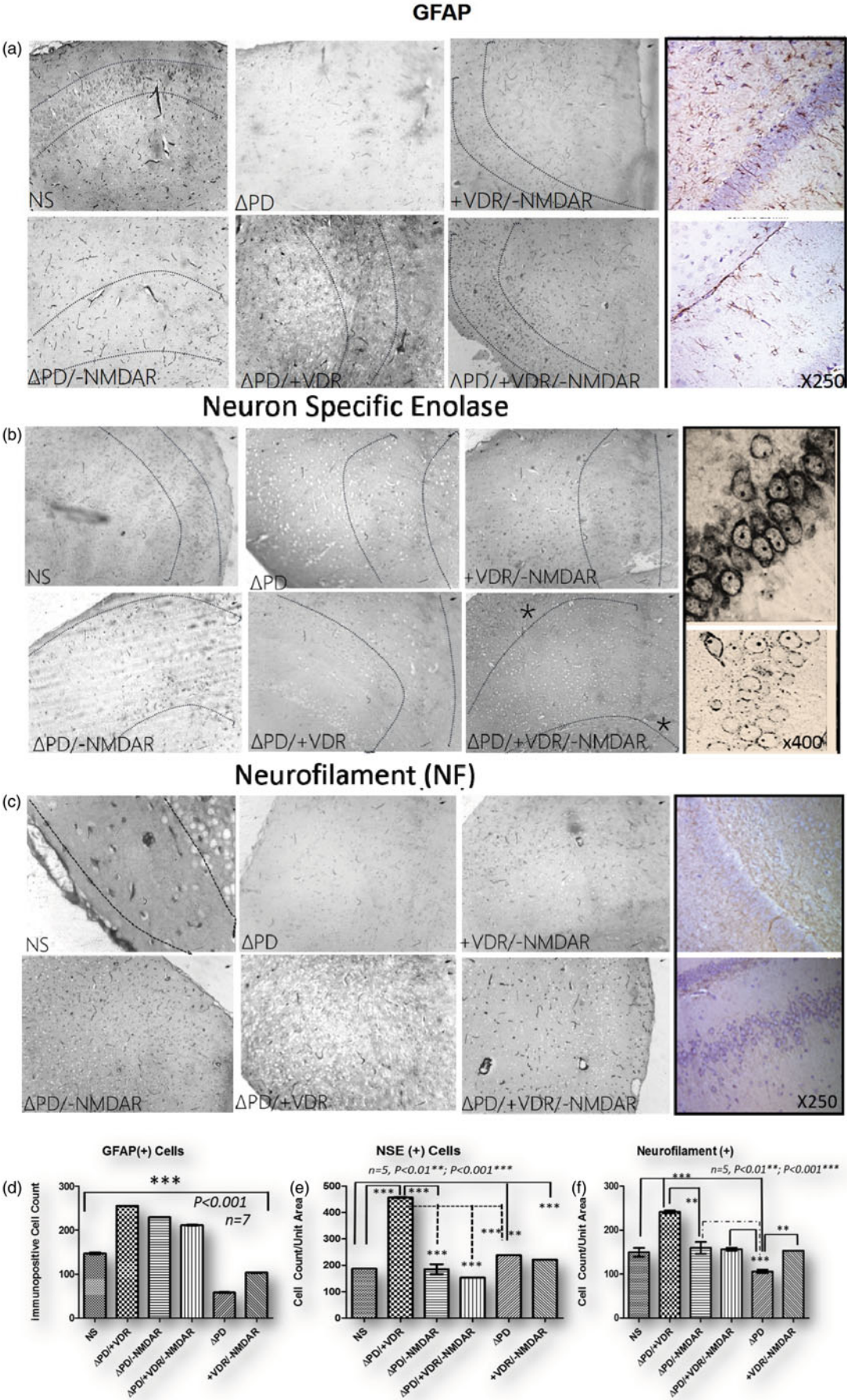


Figure 6. Expression of GFAP, NSE and NF in the motor cortex (immunohistochemistry). (a) Significant loss of glia cells was observed in the haloperidol treatment group ( $\Delta$ PD;  $p < 0.001$ ) and the animals that received intervention without prior haloperidol treatment (+VDR/-NMDAR; Continued

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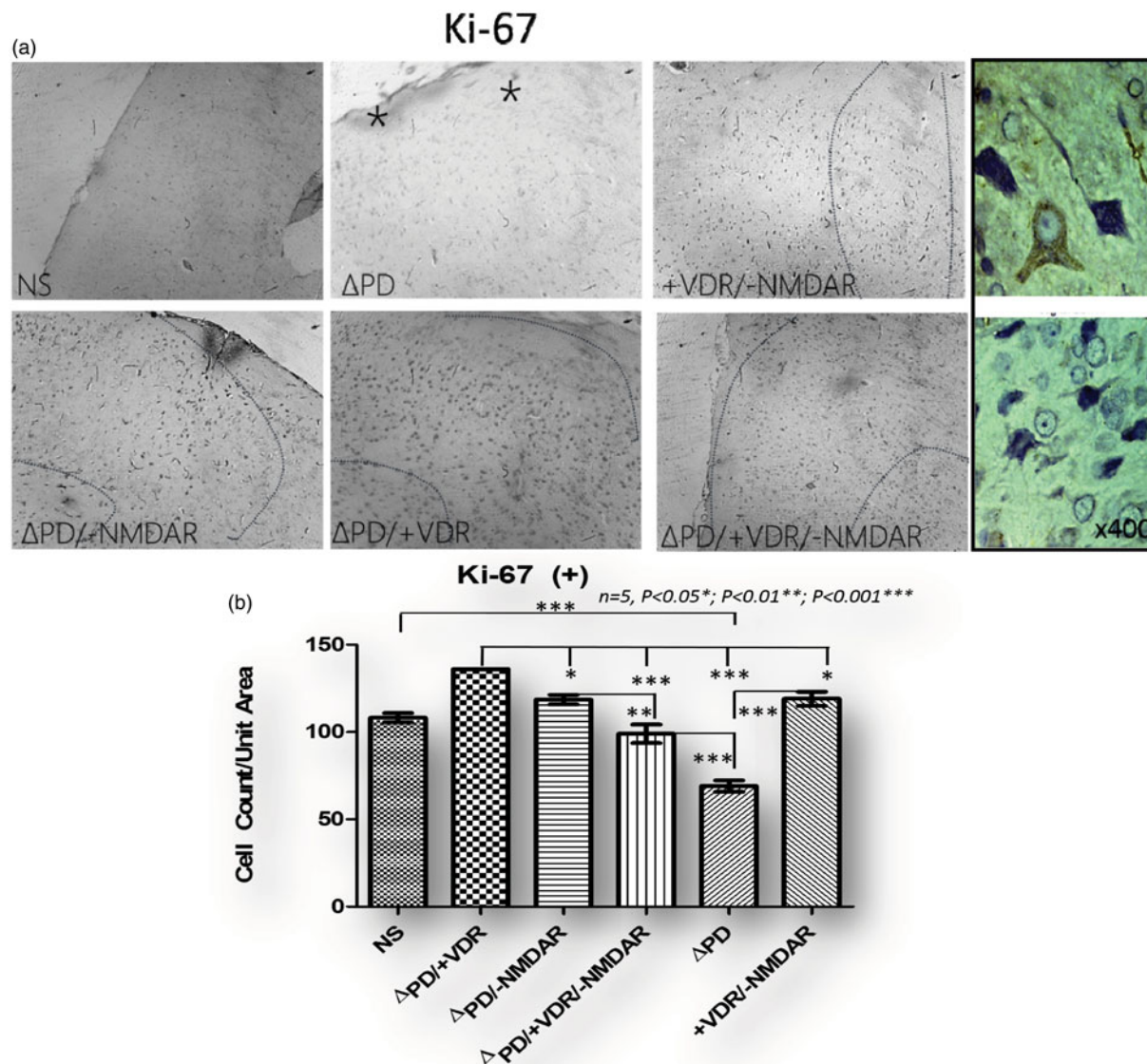


Figure 7. Ki-67 immunohistochemistry. (a) Ki-67 expression in the cortex demonstrates proliferation in the treatment groups following administration of Vitamin D<sub>3</sub> (ΔPD/+VDR). ΔPD showed the least count in Ki-67 expression when compared to the control. However, this increased significantly after the VDRA intervention ( $p < 0.001$ ), NMDAR inhibition ( $p < 0.001$ ) and combined intervention ( $p < 0.001$ ). (b) Statistical analysis of distribution of Ki-67 immunopositive cells in the motor cortex. The ΔPD/+VDR group gave the highest score while the ΔPD had the lowest count (significant at  $p < 0.001$ ). ΔPD/+VDR/-NMDAR treatment also facilitated proliferation but was less significant when compared to ΔPD/+VDR ( $p < 0.01$ ) (magnification  $\times 100$ ). [Inset black frame shows higher magnification for cell identification; \*represents region of notable cell loss in Figure 7a].

#### Figure caption Continued

$p < 0.001$ ) when compared with the control. An increase in glia count (GFAP+) was observed in the VDRA intervention (ΔPD/+VDR), NMDAR blockade (ΔPD/-NMDAR) and combined intervention (ΔPD/+VDR/-NMDAR) groups. This demonstrates the effect of the intervention in glia survival and activation for metabolic support and neurotrophic factors production (see also (d)). (b) Depletion of neurons was observed after haloperidol treatment (ΔPD); this was seen as a reduced number of NSE immunopositive cells in the motor cortex. This is also suggestive of reduced neuronal metabolism in stress. Interestingly, animals that received intervention without prior treatment showed a similar NSE(+) count when compared with the ΔPD suggesting a possible cytotoxic effect without prior elevated cerebral calcium or toxicity. When compared with the control, neuronal cell loss in the ΔPD and +VDR/-NMDAR was significant at  $p < 0.001$ . Similar to the outcomes of glia count ((a) and (d)), neuronal count also increased in the motor cortex of ΔPD/+VDR when compared with the control ( $p < 0.001$ ) and other treatment groups ( $p < 0.001$ ). However, NMDAR inhibition (ΔPD/-NMDAR) and combined intervention (ΔPD/+VDR/-NMDAR) gave no significant change in NSE(+) count when compared with the control and ΔPD (e). (c) Loss of neurofilament was observed in the motor cortex of the haloperidol treated groups (ΔPD) when compared with the control ( $p < 0.001$ ). Similar to our observations in neuron and glia counts, VDRA intervention also improved neurofilament deposition and cytoskeletal repair in the cortical cells. An increase in NF was observed in the ΔPD/+VDR when compared with the haloperidol treatment without intervention (ΔPD) ( $p < 0.001$ ). ΔPD/-NMDAR and ΔPD/+CDR/-NMDAR showed significant increase in NF expression when compared with the ΔPD (see also (f)) (magnification  $\times 100$ ). [Inset black frame shows higher magnification for cell identification; \*represents region of notable cell loss in Figure 6b].



concentration and the associated excitotoxicity. Furthermore, the VDRA is a steroid (calcitriol) capable of crossing the nuclear membrane to promote gene expression and transcription of proteins for cellular repair and metabolic shifts required for cell survival. To demonstrate cell viability following VDRA intervention, Ki-67 was quantified using stereologically. We observed a significant increase in Ki-67(+) cells in the motor cortex after VDRA intervention in the haloperidol treatment group (Figure 7a and b) ( $p < 0.001$ ). Comparing this with the counts from NSE and GFAP, we observed that glia proliferation was more than neuronal proliferation in the motor cortex. The Ki-67 score in the  $\Delta$ PD cortex was significantly low and was further confirmed by comparing the Ki-67 with the GFAP and NSE count for this group.

The use of +VDR and -NMDAR in therapeutic targeting for PD proved useful in this study similar to the proposition of Liu et al. (2013). The effect of +VDR was more significant when compared with NMDAR inhibition while +VDR/-NMDAR post  $\Delta$ PD also improved cognitive and motor function in the experimental model. Motor function increased in the  $\Delta$ PD/+VDR treatment with an increased latency of fall and reduced passive rotations in the Rotarod test. This was also supported by the improved neural activity in the motor cortex *in vivo*, with maximum peaks similar to the control (Figure 1). The +VDR/-NMDAR group that received intervention without prior haloperidol treatment also showed a decline in forced motor activity on rotarod and an increased number of passive rotations (Figure 3b). This was further supported by the reduced neural activity recorded for this group (Figure 1c) – slightly higher than those recorded for the  $\Delta$ PD (Figure 1b and g). Similarly, memory function was highest in the VDRA intervention group (Figure 4a and b) when compared with the control and the NMDAR inhibition group ( $\Delta$ PD/-NMDAR). Comparing the memory index, the  $\Delta$ PD explored the old object more than the new object, thus a decline in memory index when compared to the control. After VDRA treatment in the  $\Delta$ PD/+VDR, the memory index increased as the animals explored the new object more and recorded an increase in exploration time of both objects. The  $\Delta$ PD/-NMDAR gave only a minor improvement in memory index when compared with the  $\Delta$ PD/+VDR for the same intervention period. The reduction in neural depolarization potential observed in haloperidol treatment shows the effect of synaptic denervation in prolonged exposure to drugs, radicals or calcium surge at the synaptic endings (Liu et al., 2010). Although VDRA intervention improves cortical function, the actual mechanism of VDR-VDRA linked cytoskeletal restoration in Parkinsonism is an area to be explored in drug development.

## Conclusion

VDR activation and NMDAR inhibition improved motor functions following haloperidol induced degeneration and cell loss in the motor cortex. The VDRA treatment was more effective as demonstrated in the behavioral studies and *in vivo* neural activity recordings in the motor cortex. This was further supported by demonstration of neuron survival marker (NSE) and the presence of glia cells (GFAP) in the cortex to

produce growth factors to aid survival of neurons after VDRA intervention. Ki-67 staining further supports the findings that +VDR increases the rate of neuron and glia survival and proliferation in the motor cortex. We conclude that improved motor function following VDRA intervention is associated with an improved neural activity and cytoskeletal deposition modulated by VDR-VDRA through the reduction of intracellular calcium. In addition, VDRA activation also facilitated increased protein synthesis for metabolic support (NSE) and synaptic repair (NF).

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## Declaration of interest

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Supplementary material available online  
Supplementary Tables S1 and S2.