



Article

Neurotoxic Effect of Manganese and Vanadium Co-Exposure in Animal Models of Parkinson's Disease

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Abstract

Chronic environmental exposure to mixtures of heavy metals like manganese (Mn) and vanadium (V) has been associated with Parkinson's disease (PD). We investigated the poorly understood neurotoxic effects of Mn/V co-exposure on PD-relevant behavioral phenotypes in transgenic mice expressing the human alpha-synuclein (α Syn) A53T mutant. C57BL/6 wild-type (WT) and transgenic A53T mice were intranasally co-exposed to 100 μ g MnCl₂ and 75 μ g V₂O₅ five times weekly for three months, simulating a 5-day workweek. This led to significant Mn/V accumulation in the brain. Exploratory locomotor activity declined significantly in Mn/V-treated A53T mice, but not in Mn/V-treated WT mice when compared to their respective vehicle controls. Motor coordination, assessed via a forced locomotor activity test, was not significantly affected in either group. In Mn/V-treated A53T mice, olfactory deficits were present, but not in Mn/V-treated WT mice. Behavioral despair, assessed by tail suspension and forced swim tests, was not induced by Mn/V co-exposure in any group compared to their vehicle controls. Mn/V-treated A53T mice exhibited anxiety-like behavior and hyperactivity. These findings suggest that Mn/V co-exposure exacerbates neurotoxic effects in A53T mice, with a more pronounced effect in males, providing insight into the role of metal mixture exposure in environmentally linked Parkinsonism.

Keywords: metal exposure; mixtures; manganese; vanadium; α -synuclein; Parkinsonism



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1. Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder characterized by the degeneration of dopaminergic neurons in the substantia nigra pars compacta and the accumulation of alpha-synuclein (α Syn) protein aggregates, forming intraneuronal inclusions known as Lewy bodies. The loss of these neurons leads to reduced dopamine production in the striatum, causing hallmark motor symptoms, such as tremors, rigidity, bradykinesia, and postural instability [1–3]. In addition to these motor symptoms, a range of non-motor symptoms, including autonomic dysfunction, sensory impairments (e.g., hyposmia), cognitive deficits, sleep disturbances, and neuropsychiatric disorders (e.g., depression and anxiety), are also common in PD and can appear decades before motor symptoms [1,4–9].

While genetic factors play a major role in familial PD, environmental exposures have emerged as significant contributors to the development and progression of the disease. Among these, occupational exposure to metals has been extensively studied as a potential trigger for PD [10–15]. Manganese (Mn) has received significant attention due to its well-established neurotoxic effects, which can lead to a condition resembling PD known as manganism or Mn-induced Parkinsonism [16–18]. Despite the similarities in clinical presentations, pathological and pharmacological differences exist between manganism and idiopathic PD [19,20]. Mn tends to accumulate primarily in the basal ganglia, especially in the globus pallidus [21–23], but the exact pathogenic mechanisms underlying its neurotoxicity remain unclear.

While dietary intake is the primary route of metal exposure for the general population, occupational exposure to high doses of Mn primarily occurs via inhalation. Workers in industries such as welding, smelting, mining, alloy manufacturing, and dry-cell battery production are at an increased risk for Mn-induced Parkinsonism [24–27]. Several neuropsychological and neurological sequelae have been documented in Mn-exposed workers, including symptoms resembling PD [25,28–34]. The neurotoxicity of metals is often studied on an individual metal basis, but many individuals are routinely exposed to mixtures of metals in certain occupational environments. Specifically, Mn exposure frequently co-occurs with exposure to other metals, particularly vanadium (V), in industries such as welding, where both metals are prevalent [35–38]. Significant amounts of Mn and V are commonly found in high-strength, low-alloy steel and ferrovanadium alloy used across military and industrial applications [39].

Like Mn, excessive exposure to V can also pose neurotoxic risks [40,41]. Occupational exposure to V primarily results from inhaling vanadium pentoxide (V_2O_5) dust in various industrial environments, including petroleum and coal refineries, as well as in ferrovanadium and steel production [42]. Long-term occupational and environmental exposure to V has been associated with a range of adverse neurological effects [43]. Animal studies, including our own, have reported behavioral deficits from V exposure, such as impaired locomotion, olfactory deficits, and learning and memory problems [44–50]. Additionally, inhalation of V_2O_5 has been shown to damage the nigrostriatal system and other areas within the central nervous system [51–53]. Human studies also indicate that occupational exposure to V can result in cognitive and motor deficits, along with emotional disturbances [37,54].

Despite growing knowledge of the individual neurotoxic effects of Mn and V exposure, the interactions between these metals and their combined impact on neurotoxicity remain largely unexplored. This study aims to characterize neurobehavioral deficits following intranasal administration of an Mn and V mixture in a transgenic (Tg) mouse model of PD that overexpresses the A53T mutant form of human α Syn. The goal was to assess whether metal exposure, in combination with a genetic mutation, accelerates the onset of clinical symptoms. To test this hypothesis, young adult α Syn A53T Tg mice with no basal signs of behavioral disease symptoms were used. These mice express human A53T α Syn under the control of the mouse prion protein promoter, resulting in α Syn pathology that mirrors human PD, including α Syn aggregation, fibril formation and truncation, phosphorylation, and ubiquitination, as well as progressive age-dependent neurodegeneration [55,56]. The accumulation of α Syn in these mice results in severe motor impairments, typically manifesting after 8 months of age [55,57,58].

2. Results

2.1. The Effects of Chronic Mn/V Treatment on Locomotor Activity and Anxiety-like Behavior in α Syn A53T Tg and C57BL/6 WT Mice

The α Syn A53T Tg mice and C57BL/6 WT control mice were intranasally administered with either a Mn/V (100 μ g/75 μ g) mixture or vehicle control 5x/wk for 3 mo. To characterize the neurotoxic effects of chronic Mn/V co-exposure on neurobehavioral deficits, we first examined the locomotor activities using two well-known behavioral tests: the open-field locomotor test and the rotarod performance test. Overall, open-field locomotor activities decreased in mice, specifically α Syn A53T Tg mice, treated with a chronic dose of Mn/V for 3 mo as compared to vehicle-treated mice, as indicated by the representative ($n = 3$) activity track maps (Figure 1A) and heat maps of overall activity (Figure 1B). Interestingly, in these maps, Mn/V-treated mice spent more time near the less-exposed walls (left and bottom sides of the arena) than vehicle control groups in α Syn A53T Tg mice and male C57BL/6 WT mice, suggesting increased anxiety in Mn/V-treated groups. Furthermore, the total time spent in the most exposed corner was significantly reduced in Mn/V-treated male α Syn A53T Tg mice compared to vehicle-treated control mice (Figure 1C), while Mn/V-treated female α Syn Tg mice and Mn/V-treated male C57BL/6 mice showed a nonsignificant decrease in time spent in the most exposed corner, suggesting heightened anxiety in these Mn/V-treated groups. In contrast, Mn/V-treated female C57BL/6 WT mice spent more time in the most exposed corner than their vehicle-treated counterparts (Figure 1C).

In addition, our results show significantly reduced horizontal and vertical movements and increased time immobile at Month 3 in Mn/V-treated male α Syn A53T Tg mice compared to vehicle control (Figure 1E–G). However, the reduction in the total distance traveled by Mn/V-treated male α Syn A53T Tg mice was not significant (Figure 1D). Mn/V-treated female α Syn A53T Tg mice also show significantly reduced vertical movements at Month 2, while horizontal movements, total distance, and time immobile were not significantly altered after Mn/V treatment (Figure 1H–K). Mn/V treatment did not significantly affect any of the four locomotor parameters in C57BL/6 WT mice compared to their vehicle-control group (Figure 1L–S). Moreover, the representative velocity graphs ($n = 3$) demonstrate a decrease in velocity and movement, especially in α Syn A53T Tg mice and male C57BL/6 WT mice (Figure 2A). The within-strain comparisons between Mn/V- and vehicle-treated groups suggest that the susceptibility to locomotor deficits following chronic Mn/V treatment is heightened in α Syn A53T Tg mice. However, vehicle-treated α Syn A53T Tg mice exhibit increased exploratory locomotor activity in the open-field test, as observed in the track plots (Figure 1A), heat maps (Figure 1B), total distance traveled (Figure 1D,H,L,P), and velocity graphs (Figure 2A), showing that these mice are more hyperactive and show reduced anxiety-like behavior compared to vehicle-treated C57BL/6 WT mice.

To detect impairments in forced locomotor coordination, we subjected mice to three 3-min trials in a constant-acceleration paradigm on the rotarod (AccuScan). Mn/V treatments did not lead to any significant motor coordination or motor skill learning deficits in either α Syn A53T Tg or C57BL/6 WT mice performing the rotarod test over the 3-mo study (Figure 2B–E). This is explained by the observation that Mn/V-treated mice gradually lost weight or maintained a constant weight, whereas vehicle-treated mice gradually gained weight, such that Mn/V-treated and vehicle-treated α Syn A53T Tg mice differed significantly in weight (Supplementary Figure S1A–D). This weight differential could produce differences in agility that offset mild impairments of vestibular coordination. The effects of this weight differential can also be seen when contrasting the rotarod performance of vehicle-treated heavier males vs. lighter females (Supplementary Figure S1E,F). Similar to the open-field test, vehicle-treated α Syn A53T Tg mice demonstrated better balance and

motor coordination, as indicated by a longer latency to fall off the rotarod (Figure 2B–E and Supplementary Figure S1F). This supports the notion that these animals are hyperactive compared to the vehicle-treated C57BL/6 WT mice, potentially contributing to improved rotarod stamina and/or coordination.

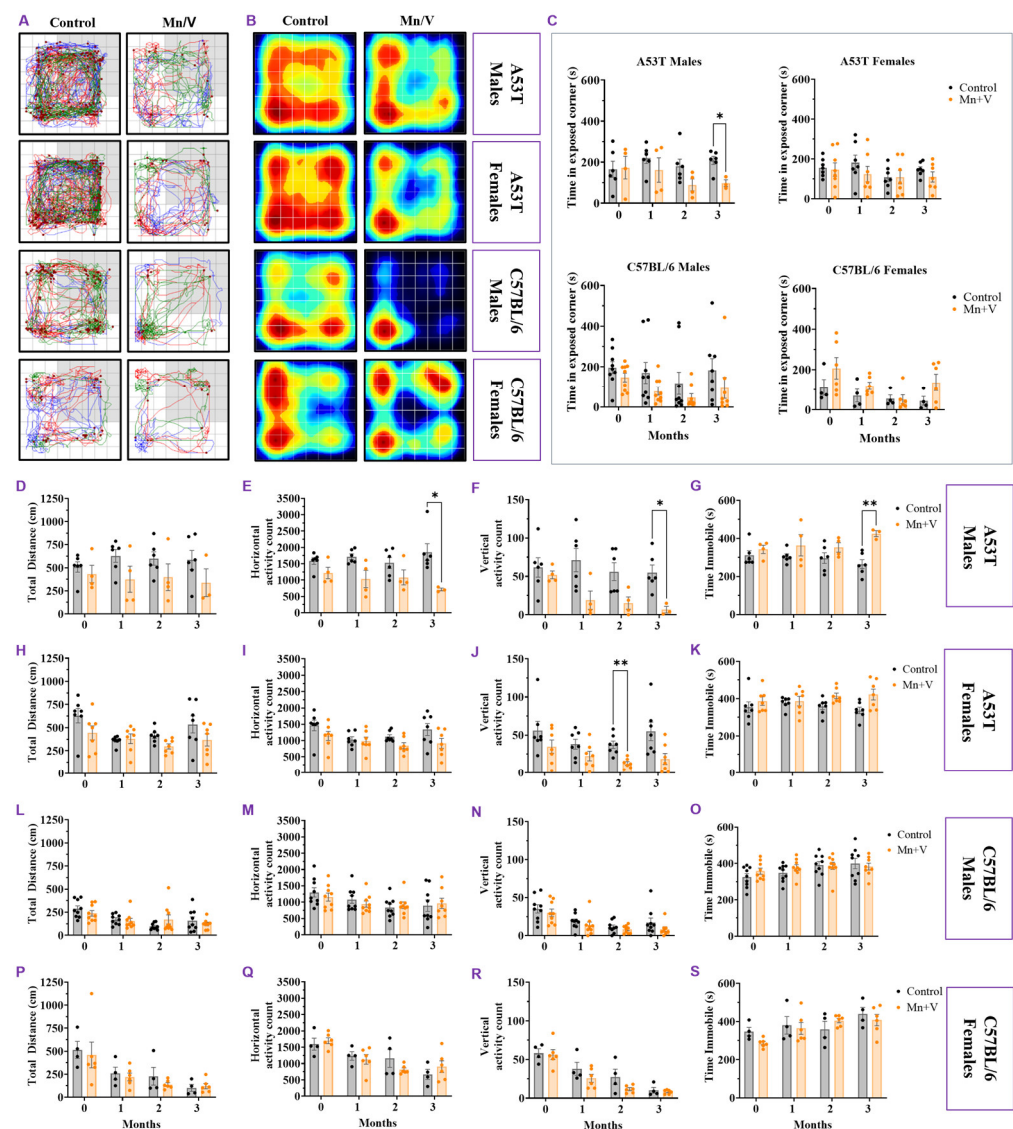


Figure 1. Effects of chronic Mn and V co-exposure on open-field locomotor activity in α Syn A53T Tg and C57BL/6 WT mice. Mice were intranasally administered a combined dose of 100 μ g Mn and 75 μ g V (orange) or vehicle control (black) 5x/week for 3 months. Locomotor activity was assessed in an open-field arena using VersaMax. (A) Representative track maps and (B) heat maps of overall activities at Month 3 are shown ($n = 3$). Mouse trajectories are displayed in different colors (blue, red, and green), with each color corresponding to an individual mouse. Heat map colors range from blue (less time spent) to red (more time spent). The bottom-left corner of the 20 $\times$ 20 cm arena had opaque walls, whereas the top-right corner (gray area in the track maps) was more exposed, with transparent walls that met in the center of the larger 40 $\times$ 40 cm chamber. Bar charts show (C) time spent in the most exposed corner (s), (D,H,L,P) total distance traveled (cm), (E,I,M,Q) horizontal activity, (F,J,N,R) vertical activity, and (G,K,O,S) time immobile (s) for each group. The x -axis represents time (months). Mouse strain and sex are indicated on the right side of each panel. Statistical significance is indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$) based on two-way ANOVA or mixed-effect analysis with Geisser–Greenhouse correction and Šidák’s multiple comparisons test.

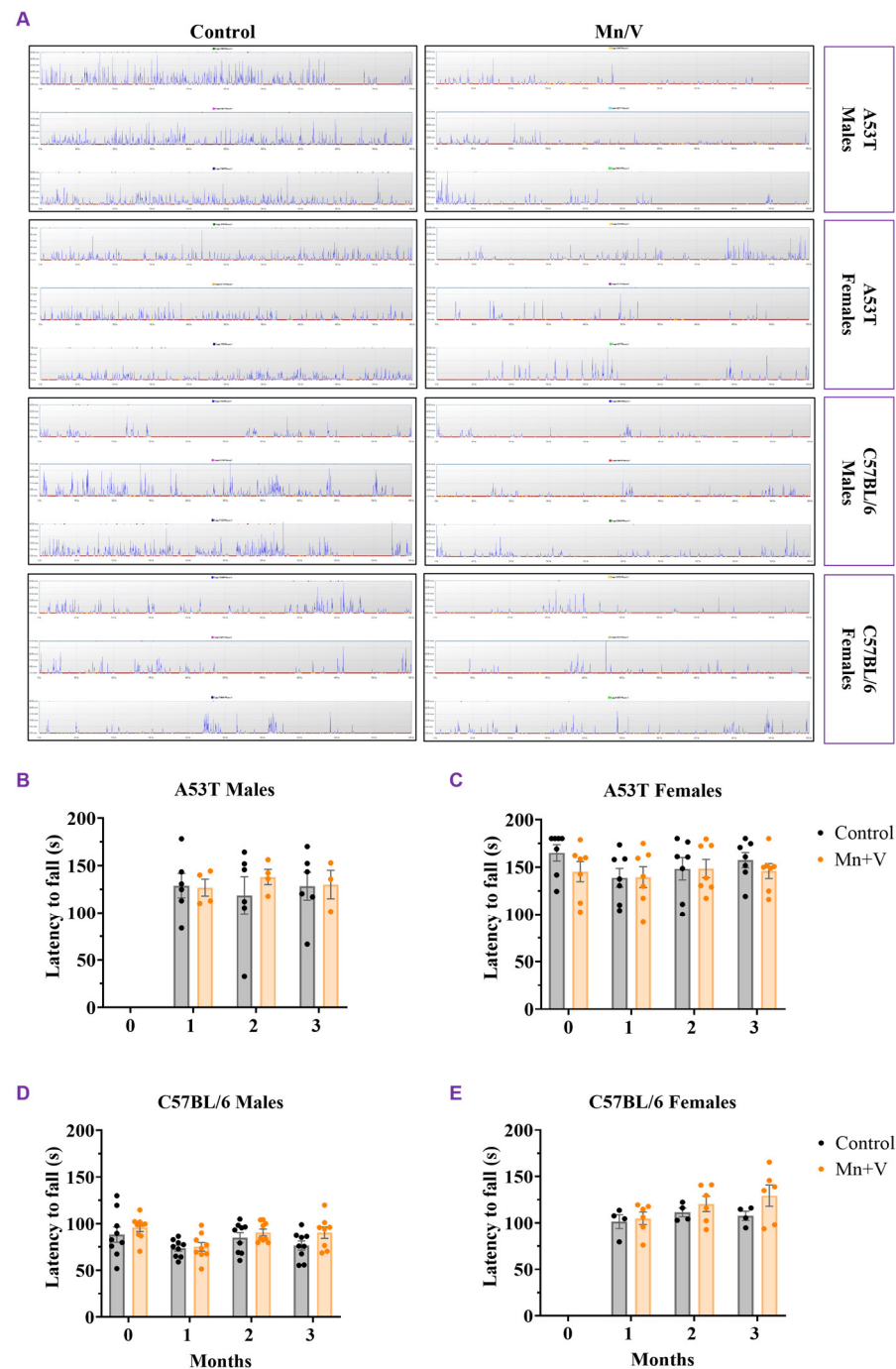


Figure 2. Effects of chronic Mn and V co-exposure on velocity, forced locomotor activity, and vestibular coordination in α Syn A53T Tg and C57BL/6 WT mice. Mice were intranasally administered a combined dose of 100 μ g Mn and 75 μ g V (orange) or vehicle control (black) 5x/week for 3 months. Velocity was assessed using the open-field test, while forced locomotor activity and vestibular coordination were evaluated using the rotarod test. Latency to fall was recorded as the average of three trials. **(A)** Representative velocity traces ($n = 3$) are shown over 600 s. Y-axis velocity scales differ between panels (0–40, 0–60, or 0–100 cm/s). **(B–E)** Average latency to fall (s) is shown for each group. The x-axis represents time (months). Mouse strain and sex are indicated at the top of each graph.

2.2. The Effects of Chronic Mn/V Treatment on Olfactory Function in α Syn A53T Tg and C57BL/6 WT Mice

We used the social discrimination test to examine the neurotoxic effects of chronic Mn/V co-exposure on olfactory function. Chronic Mn/V treatment did not induce significant changes in total distance traveled, total entries, or discrimination ratio in male α Syn

A53T Tg mice (Figure 3A–C). However, the time spent in the novel scent zone by male α Syn A53T Tg mice significantly decreased at Month 3 of Mn/V treatments (Figure 3D). The time spent in the novel zone was also significantly decreased at Months 2 and 3 in Mn/V-treated female α Syn A53T Tg mice (Figure 3H), while the total distance traveled, total entries, and discrimination ratio had non-significant changes after 3 mo of Mn/V exposure (Figure 3E–G). Mn/V treatment did not induce significant olfactory dysfunction in male C57BL/6 WT mice, as measured by the total distance traveled, total entries, discrimination ratio, and time spent in the novel zone (Figure 3I–L). In female C57BL/6 WT mice, the total entries significantly decreased at Month 3 of Mn/V exposure (Figure 3N), while the time spent in the novel zone decreased over time but non-significantly (Figure 3P). The total distance traveled and discrimination ratio did not change after 3 mo of Mn/V exposure in female C57BL/6 WT mice (Figure 3M,O).

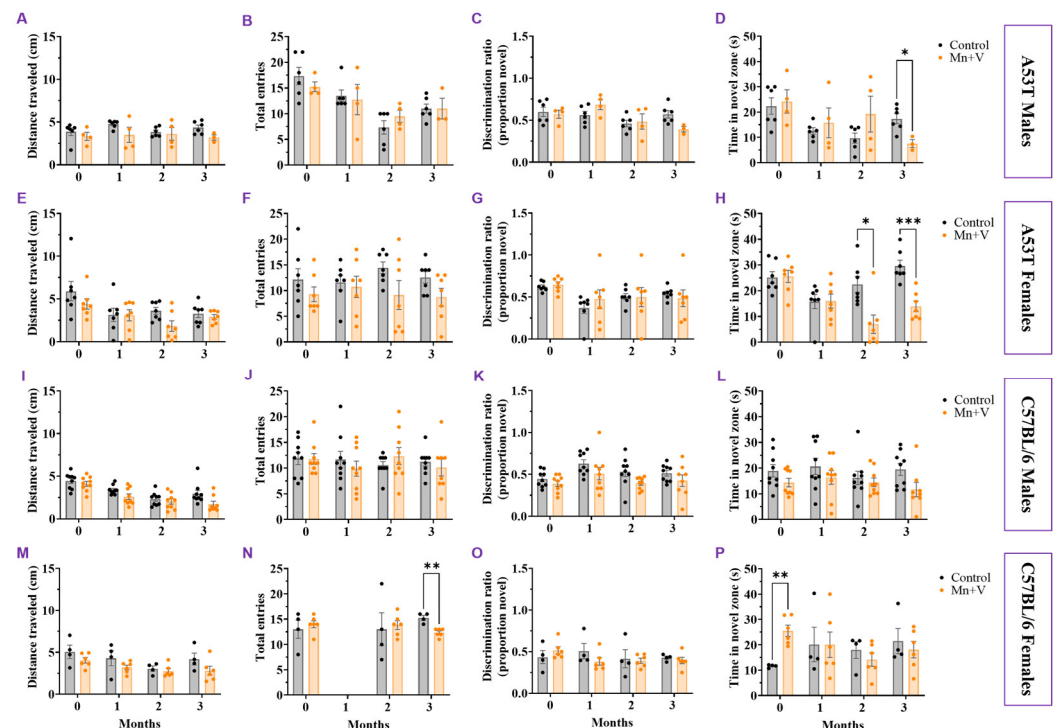


Figure 3. Effects of chronic Mn and V co-exposure on olfactory function in α Syn A53T Tg and C57BL/6 WT mice. Mice were intranasally administered a combined dose of 100 μ g Mn and 75 μ g V (orange) or vehicle control (black) 5x/week for 3 months. Olfactory function was assessed using the social discrimination test. Panels show (A,E,I,M) the total distance traveled (cm), (B,F,J,N) the number of times the mice sniff the scent source, (C,G,K,O) the proportion of entries (discrimination ratio) that are in the novel scent zone, and (D,H,L,P) the total time spent in the novel scent zone (s). The x-axis represents time (months). Mouse strain and sex are indicated on the right side of each panel. Statistical significance is indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) based on two-way ANOVA or mixed-effect analysis with Geisser–Greenhouse correction and Šidák’s multiple comparisons test.

2.3. The Effects of Chronic Mn/V Treatment on Behavioral Despair in α Syn A53T Tg and C57BL/6 WT Mice

We next assessed behavioral despair by subjecting mice to the tail suspension test (TST) and forced swim test (FST). Both inescapable tasks inevitably lead to episodes of immobility that reflect depressive behavior. Mn/V treatment did not induce significant differences in immobility between either the α Syn A53T Tg groups or C57BL/6 WT groups (Figure 4), suggesting that chronic Mn/V exposure for up to 3 mo does not induce depression-like symptoms in these groups. As with the rotarod test, sex- and treatment-induced weight

differences among groups may confound performance in these physically demanding behavioral tests. The heaviest mice (male C57BL/6 WT) tended to be the most immobile, while the lower-weight mice (female α Syn A53T Tg mice) appeared more mobile throughout these tasks (Figure 4 and Supplementary Figure S1G,H). Additionally, the immobility scores decline in vehicle-treated α Syn A53T Tg mice compared to vehicle-treated C57BL/6 WT mice at 6 mo of age (Supplementary Figure S1G,H), indicating that the mice spend more time attempting to escape. Consistent with findings from the open-field and rotarod tests, α Syn A53T Tg mice exhibited greater hyperactivity than their C57BL/6 WT counterparts.

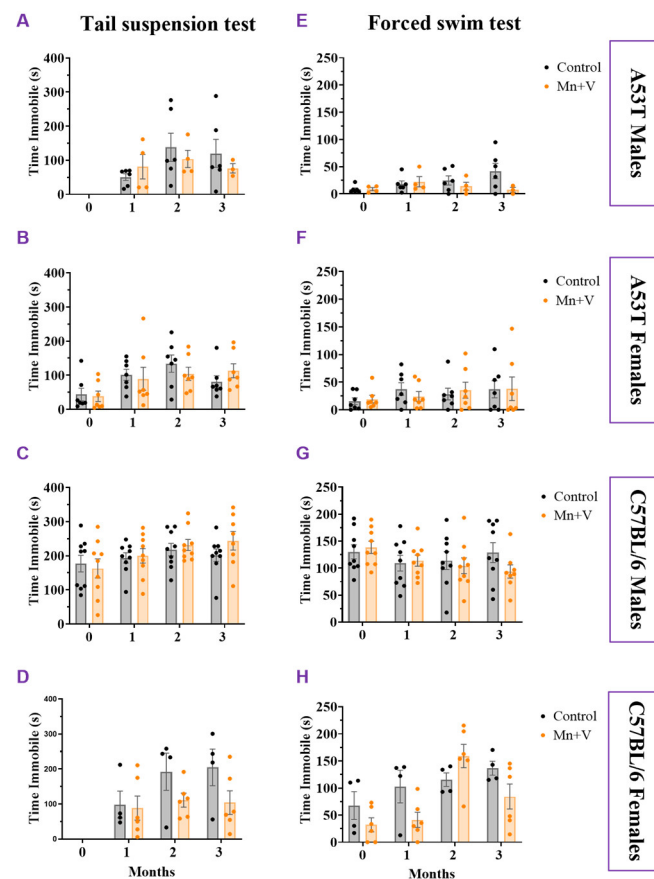


Figure 4. Effects of chronic Mn and V co-exposure on depression-like behavior in α Syn A53T Tg and C57BL/6 WT mice. Mice were intranasally administered a combined dose of 100 μ g Mn and 75 μ g V (orange) or vehicle control (black) 5x/week for 3 months. Depression-like behavior was assessed using the tail suspension and forced swim tests. The average time spent immobile (s), as an indirect measure of depression-like behavior, was plotted in the (A–D) tail suspension and (E–H) forced swim tests. The x-axis represents time (months). Mouse strain and sex are indicated on the right side of each panel.

2.4. The Effects of Chronic Treatment with Mn/V on Metal Concentrations in the Brain of α Syn A53T Tg and C57BL/6 WT Mice

We next used inductively coupled plasma mass spectrometry (ICP-MS) to determine whether long-term intranasal Mn/V treatment resulted in metal uptake into the brain. Relative to vehicle-treated controls, the brain concentration of Mn significantly increased after 3 mo of chronic Mn/V co-exposure in both α Syn A53T Tg and C57BL/6 WT mice (Figure 5A,C,E,G). In contrast, the brain concentration significantly increased in male α Syn A53T Tg and male C57BL/6 WT mice (Figure 5B,F), while female α Syn A53T Tg and female C57BL/6 WT mice showed only non-significant increases (Figure 5D,H). These results

suggest that chronic intranasal Mn/V co-exposure for 3 mo leads to accumulation of Mn and V in the brain.

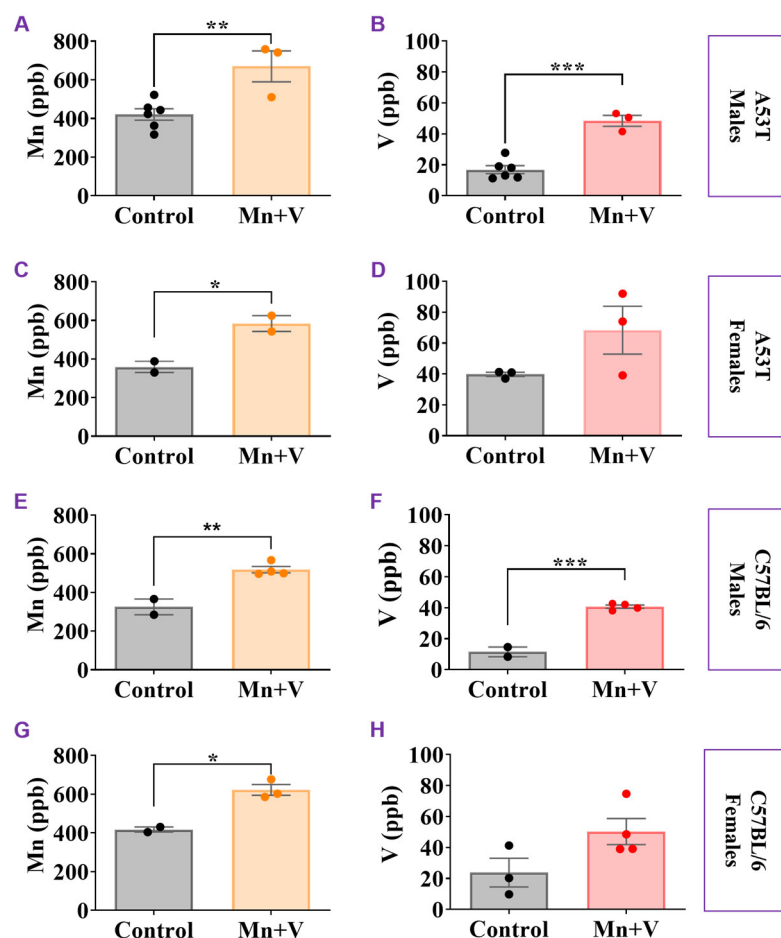


Figure 5. Effects of chronic Mn and V co-exposure on the brain of α Syn A53T Tg and C57BL/6 WT mice. Mice were intranasally administered a combined dose of 100 μ g Mn and 75 μ g V or vehicle control 5x/week for 3 months. Plots show brain concentrations of (A,C,E,G) manganese (Mn) and (B,D,F,H) vanadium (V) in parts per billion (ppb), as determined by ICP-MS. Mouse strain and sex are indicated on the right side of each panel. Statistical significance is indicated by asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$) based on a two-tailed unpaired t-test assuming a Gaussian distribution.

3. Discussion

This study demonstrates that Mn and V accumulate in the brain following three months of chronic intranasal co-exposure to these metals, resulting in impaired motor and olfactory performance, as well as increased anxiety in α Syn A53T Tg mice. These findings suggest that combining genetic alterations with exposure to a mixture of neurotoxic metals may synergistically accelerate the development of PD symptoms. This is the first study, to our knowledge, demonstrating the adverse effects of Mn and V co-exposure on neurobehavioral performance in a PD rodent model.

Some metals, including Mn, iron (Fe), copper (Cu), and zinc, are essential nutrients that play vital roles in various biochemical and physiological functions necessary for maintaining normal health; however, excessive exposure to these metals may result in toxic effects. In contrast, other metals commonly encountered in occupational settings, such as V, are primarily considered toxic and may exert adverse health effects even at relatively low exposure levels. Many individuals are routinely exposed to mixed metals in certain occupational settings through inhalation of fumes generated during welding or smelting [25,26]. Welding fumes consist of complex mixtures of metals originating from

base materials, including Mn and Fe, which are present in all types of steel, as well as alloys or residual elements added to enhance steel properties, such as V, Cu, lead, and nickel [39,59,60]. Since human exposure to Mn and V is more likely to occur via inhalation, we investigate the neurotoxic effects of these two metals through intranasal co-exposure in a mouse model of PD mimicking a 5-day workweek of occupational exposure.

We demonstrate that intranasal Mn/V co-exposure for three months leads to increased levels of Mn and V in the brain of both α Syn A53T Tg mice and C57BL/6 WT mice. While the body can efficiently remove excess Mn, mainly through the gut and liver, the brain cannot, as its elimination rate is estimated to be slower [61]. This may contribute to pathological Mn accumulation and subsequent neurotoxic effects. Mn enters the brain via three routes: the blood–brain barrier (BBB), the blood–cerebrospinal fluid (CSF) barrier, and the olfactory tract [62]. The BBB and the blood–CSF barrier are the two primary interfaces that regulate brain Mn homeostasis after oral uptake [63,64]. Unlike ingested Mn, inhaled Mn can be directly transported into the brain via the olfactory neuroepithelium, circumventing the BBB and blood–CSF barrier [65–69]. In addition to Mn, a previous study showed that V can enter the brain parenchyma following inhalation exposure in a mouse model and that V alters the structure of the ependymal epithelium, leading to BBB disruption [53]. According to a time-course study, the half-life of V elimination in V-fed rats is approximately 12 days [70]. Hence, chronic inhalation of both Mn and V can lead to their accumulation in the brain, as our results demonstrate. Notably, the accumulation of Mn and V in various regions of the human brain, along with 49 other elements, has been previously reported, supporting the relevance of our findings to human exposure [71].

Although the neurotoxicity of metals is commonly studied on an individual metal basis, humans are routinely exposed to mixtures of metals in certain environments. Little is known about how Mn and V interact in mixtures and how these interactions cause metal-induced neurotoxicity. Our previous *in vitro* study demonstrated that Mn and V co-exposure produces a more potent neurotoxic response than exposure to either metal alone under similar conditions (unpublished data), and the *in vivo* study also showed that Mn/V co-exposure induces severe neurotoxicity in the olfactory system [45]. Here, we investigate the neurotoxic effects of Mn/V co-exposure on neurobehavioral performance in a mouse model of PD, in which the A53T mutant form of human α Syn is overexpressed. These mice exhibit several clinicopathological features of PD, including motor dysfunction [56,72], accumulation of Lewy bodies in the neocortex, hippocampus, and substantia nigra, and a loss of dopaminergic terminals in the basal ganglia [72]. In this mouse PD model, the human α Syn A53T mutant, not the endogenous mouse α Syn, aggregates and contributes to neurodegenerative pathology [55].

Our open-field test results suggest that susceptibility to motor deficits following chronic Mn/V treatment is heightened in α Syn A53T Tg mice, particularly male mice, compared to their vehicle control group. This effect was not seen in C57BL/6 WT mice. The vertical activity (rearing) count indicates the number of times the mice stand on their hind legs, often recorded as a measure of active exploration. Therefore, reduced rearing may suggest reduced motivation to explore its surroundings by sight and smell. Mn/V-treated α Syn A53T Tg mice exhibited decreased vertical activity counts in both males and females. In addition, Mn/V-treated male α Syn A53T Tg mice exhibited decreased overall activity, characterized by reduced horizontal activity and increased time immobile, potentially indicating motor impairment. On the other hand, forced locomotion on a rotarod did not show any significant effects on vestibular coordination after Mn/V co-exposure for three months compared to the vehicle control group in either α Syn A53T Tg mice or C57BL/6 WT mice. We cannot exclude the possibility that the continuous weight loss in Mn/V-treated animals made them more agile, thereby counteracting other developing effects potentially

induced by Mn/V co-exposure. Rotarod performance has been reported to be affected by differing body weight, with lower-weight mice often exhibiting prolonged latencies, while heavier mice tend to show reduced latencies due to difficulty maintaining balance on the rod [73]. Additionally, open-field and rotarod testing revealed that vehicle-treated α Syn A53T Tg mice performed better overall than vehicle-treated C57BL/6 WT mice, with greater distance traveled throughout the arena and longer latency to fall from the rotarod. These findings are consistent with previous studies, which have shown that enhanced exploratory and locomotor activity is characteristic of A53T mutant mice [58,74,75]. This behavior pattern in α Syn A53T Tg mice shows a trend toward reduced anxiety-like behavior and increased hyperactivity, as reported in the literature [74,75].

Recognition of the important role of various non-motor symptoms in prodromal and early-stage PD [1,4] has instilled a renewed emphasis on pre-motor symptoms to detect PD early enough to improve prognosis and disease control. Thus, we monitored clinically relevant non-motor symptoms in α Syn A53T Tg and C57BL/6 WT mice for three months of Mn/V co-exposure. One of the earliest and most noticeable impairments associated with PD is hyposmia, characterized by a reduced sense of smell. Decreased olfactory function, manifesting as difficulties in odor detection, identification, and discrimination, is highly prevalent among PD patients and can occur years, even decades, before the onset of motor symptoms [68,76–78]. This emphasized the potential of olfactory screening as a prognostic tool for early detection of PD [68,76–80]. Therefore, we assessed the impact of chronic Mn/V co-exposure on olfactory function through the social discrimination test. Our results revealed significant olfactory deficits in Mn/V-treated α Syn A53T Tg mice, as indicated by a reduced time spent in the opposite-sex bedding (novel) zone. A longer time investigating the novel scent typically reflects normal olfactory processing and memory function. Conversely, lessening time spent investigating the novel scent may suggest impaired olfactory recognition or diminished interest in novelty. Thus, our findings indicate that chronic co-exposure to Mn/V exacerbates susceptibility to olfactory deficits in 6-mo-old α Syn A53T Tg mice. Supporting this, our previous research demonstrated olfactory impairments following one month of intranasal V exposure [44,45], as well as Mn and Mn/V co-exposure 3x/wk [45] in male C57BL/6 mice. Additionally, research has shown that cadmium, chromium, nickel, and Mn can adversely affect the sense of smell following prolonged exposure [68]. These findings suggest that metal exposure may contribute to olfactory dysfunction, offering a potentially valuable screening tool for identifying individuals at high risk of developing Parkinsonian syndrome due to metal neurotoxicity.

We subsequently examined whether chronic treatment with Mn/V induces neuropsychiatric symptoms in both α Syn A53T Tg and C57BL/6 WT mice. Depression is highly prevalent in PD across all stages of the disease, affecting more than half of PD patients and significantly impacting their quality of life [81–83]. To assess depressive behaviors, we conducted the TST and FST as two inescapable tasks that produce behavioral despair in the form of increasing immobility. These tests are commonly used to evaluate depression-like behavior in rodents, and the behavioral despair exhibited by rodents offers insight into affective states that may help model human depression. In these tests, longer immobility suggests a reduced motivation to escape or a tendency to give up, which is interpreted as an indicator of depressive-like behavior. Conversely, decreased immobility can reflect a healthier or less depressed state as the mice spend more time attempting to escape. Our results indicate that chronic exposure to Mn/V mixture for up to 3 mo does not lead to the development of depression-like symptoms in either α Syn A53T Tg or C57BL/6 WT mice, as demonstrated by both behavioral assessments. However, we are unable to exclude the possibility that the weight change difference observed between Mn/V-treated and vehicle-

treated mice potentially influenced the outcomes of these physically demanding tests. In addition, vehicle-control α Syn A53T Tg showed reduced depressive-like behavior relative to vehicle-control C57BL/6 WT mice. These data are consistent with a previous report by Oaks and colleagues [57], and could be explained by the hyperactivity that presymptomatic α Syn A53T Tg mice exhibit.

Like depression, anxiety is a common non-motor PD symptom that can significantly impact the quality of life for those with PD [84]. Interestingly, observations from the activity track and heat maps generated during the open-field test reveal that Mn/V-treated mice tended to spend more time near the opaque walls, a zone that offers less exposure than the transparent walls. This change in their thigmotaxic behavior suggests that Mn/V treatment may induce greater anxiety in mice, dampening their inherent propensity to explore the open-field arena along all walls [85,86]. Additionally, a decline in horizontal activity may indicate increased anxiety levels, as anxious mice are likely to spend less time in the center of the arena, avoiding open spaces and preferring to stay near the walls or corners where they feel more secure, especially if their locomotor performance is becoming impaired, resulting in increased immobility. In our findings, male α Syn A53T Tg mice treated with Mn/V exhibited significantly decreased horizontal activity and more time spent immobile. Furthermore, vertical activity can provide insight into anxiety-like behavior, as anxious mice might avoid rearing due to reluctance to leave their safe corners. Both female and male α Syn A53T Tg mice treated with Mn/V reduced their vertical activity. Overall, our open-field analysis suggests that Mn/V-treated α Syn A53T Tg mice, particularly males, may exhibit heightened anxiety levels compared to vehicle-treated controls, a finding not observed in C57BL/6 WT mice. Supporting this, another study [87] demonstrated significant anxiety-like behavior in male adult mice following six months of chronic arsenite exposure via drinking water, as assessed by the open-field test.

This study has several limitations, including small and unequal group sizes and animal loss during the study, which reduces statistical power and the ability to detect small effects. In addition, baseline measurements were not available for certain behavioral outcomes in specific groups; therefore, all outcomes were analyzed using between-group comparisons at the test time point. However, randomization and standardized testing conditions were applied to minimize potential bias.

In conclusion, chronic Mn/V co-exposure significantly exacerbates motor deficits, odor impairments, and anxiety-like behaviors in α Syn A53T Tg mice, in contrast to C57BL/6 WT mice. However, exposure to these metals for up to three months does not produce depression-like symptoms in either group, as assessed by the tail suspension and forced swim tests. Additionally, intranasal administration of the Mn/V mixture resulted in the accumulation of these metals in the brain. These findings underscore the detrimental impact of Mn/V co-exposure on Parkinsonism progression in α Syn A53T Tg mice, with male mice showing a more pronounced effect than females. Further studies are needed to investigate the biochemical and histological mechanisms underlying Mn/V-induced neurotoxicity. Long-term and dose-response studies in both sexes, as well as evaluation of potential protective or therapeutic interventions, could provide valuable insights into mitigating the effects of occupational or environmental metal exposure.

4. Materials and Methods

4.1. Chemicals and Reagents

MnCl₂ (CAS Number:13446-34-9) and V₂O₅ (CAS Number: 1314-62-1) were purchased with purities of 99.99% from Sigma Aldrich (St. Louis, MO, USA). Standards for elemental analysis for the inductively coupled plasma mass spectrometry (ICP-MS) were obtained from Inorganic Ventures (Christiansburg, VA, USA), while digestion vessels, trace mineral

grade nitric acid, and hydrochloric acid were obtained from Fisher Scientific (Pittsburgh, PA, USA).

4.2. Animals and Treatment Paradigm

Animal care and procedures strictly followed the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) at Iowa State University (IACUC Protocol#: 18-309). A total of 52 mice were randomly assigned to eight experimental groups (male or female; α Syn A53T Tg or C57BL/6 WT; Mn/V or vehicle control), matched for age and weight; group sizes ranged from 4 to 9 mice. We determined a sample size of six, but in some cases, it was reduced due to mortality or mouse unavailability. The α Syn A53T Tg mice (B6;C3-Tg(Prnp-SNCA*A53T)83Vle/J), which express high levels of the human A53T mutant form of α Syn in the brain, were purchased from Jackson Laboratory (Bar Harbor, ME, USA), and non-Tg mice (WT C57BL/6NCtrl (027)) were purchased from Charles River (Wilmington, MA, USA). All mice were housed individually in ventilated cages at room temperature, under a 12-h light/dark cycle, in a humidity-controlled environment. Food and water were provided ad libitum. Environmental enrichment was provided in the form of nesting material and a plastic pipe shelter. The animals were monitored daily and weighed weekly. Mice were euthanized if they exhibited $\geq 20\%$ loss of baseline body weight or signs of severe distress or lethargy. Two mice were euthanized prior to study completion due to $\geq 20\%$ loss of baseline body weight, one of them having signs of vestibular rolling.

MnCl₂ and V₂O₅ (Mn/V) were administered intranasally at doses of 100/75 μ g per mouse. Male and female α Syn A53T Tg mice (3-4-mo-old) and female C57BL/6 WT mice (4-5-mo-old) received 20 μ L solution, while male C57BL/6 WT mice (5-mo-old) received 10 μ L to prevent leakage while maintaining the same metal concentration. Vehicle control groups received an equal volume of deionized water (pH $\sim$ 7.2–7.4). The intranasal administration was performed using micropipettes after briefly anesthetizing the mice with isoflurane to minimize distress and ensure accurate dosing, following the order determined by randomization (e.g., control, treatment, control, control, treatment, etc.). Treatments were administered 5x/wk for 3 mo, simulating a 5-day workweek of occupational exposure. Intranasal delivery was chosen to mimic human occupational exposure to airborne mixed metals via inhalation.

Treatment and behavioral testing were preceded by an acclimation period, during which mice were familiarized with each behavioral device to stabilize performance before baseline sessions and post-treatment testing. During the 3 mo of Mn/V exposure, all mice underwent a monthly battery of behavioral tests, including measurements of locomotor activity, olfactory function, depression- and anxiety-like behaviors. Behavioral testing was conducted at the same time of day and under consistent environmental conditions by the same trained personnel to minimize variability. Coded animal numbers provided objective data collection, minimizing bias, even though the investigators performing all procedures were aware of treatment allocation for dosing and data organization for analysis and graphing. Cage positions were kept constant throughout the study; although this could represent a potential confounder, standardized handling, randomization, and controlled testing conditions were applied to reduce bias. Bimonthly submandibular blood collection, alternating serum and plasma samples, was performed to assess molecular endpoints. Animals were sacrificed at the end of the 3-mo exposure period for blood collection via cardiac puncture and brain tissue dissection for neurochemical and biochemical analyses, including quantification of brain metal accumulation by ICP-MS.

4.3. Locomotor Activity

A computerized infrared activity monitoring system (VersaMax monitor, model RXYZCM-16; analyzer, model VMAUSB; AccuScan Instruments Inc., Columbus, OH, USA) was used to assess spontaneous exploratory locomotor behavior in the open-field activity test as previously described [44,88,89]. The activity chamber in the open-field test is made of transparent Plexiglas ($40 \times 40 \times 30.5$ cm) and is covered with a ventilated Plexiglas lid. Infrared (IR) monitoring sensors are located every 2.54 cm along the chamber perimeter, 2.5 cm above the floor (16 IR beams along each side). On two opposite walls, a second row of IR sensors is located 8.0 cm above the floor to detect vertical rearing. In this open-field apparatus, the larger chamber can be partitioned into four arenas, thus permitting two mice to be tracked simultaneously in two diagonally opposed ($20 \times 20 \times 30.5$ cm) arenas. Two sides of each partitioned arena have opaque walls (less exposed zone), while the other two sides have transparent walls (more exposed zone) that meet at the center of the larger chamber. Mice were monitored for time spent in the most exposed corner (s), total distance traveled (cm), horizontal activity count, vertical activity count, time immobile (s), and velocity (cm/s); activity counts refer to the number of IR beam breaks. The first 2 min of a 12 min session were allocated to acclimatization to reduce the novelty effect, leaving the last 10 min for analysis.

The rotarod (AccuScan) performance test uses forced locomotion to assess vestibular coordination as previously described [88]. In this test, the speed was set to a constant acceleration paradigm that began at 4 rpm and increased to 60 rpm at a rate of 20 rpm/min. This paradigm challenges the vestibular system in maintaining balance, orientation, and spatial awareness. The average latency to fall from the rod was measured for up to 3 min across three trials. Passive rotation (360 degrees) was interpreted as a loss of control and counted as a fall, with the experimenter manually tripping the fall sensor to end the trial. A series of 3 acclimation training days was conducted to allow the animals to reach plateau performance levels before testing began.

4.4. Social Discrimination

The effects of chronic Mn/V co-exposure on olfactory function were examined using a social discrimination test as previously described [44,89]. As a measure of olfactory performance, this test assessed the animal's ability to detect a novel scent when presented with self-bedding (familiar scent) vs. opposite-sex (novel scent) bedding during a 3 min session. The bedding was placed inside two round enclosed containers with an opening at the center of the top. The zones were marked based on the animal's head being within a defined zone (1 cm perimeter around the bedding container). The two bedding containers were fixed at either end of an opaque rectangular box before inserting the mouse in the middle, facing the self-bedding zone. This test was video-tracked from above and analyzed using ANY-maze software (v6.2; Stoelting, Wood Dale, IL, USA). The data were plotted based on the total number of entries (sniffing episodes, representing the number of times each mouse enters a sniff zone to presumably sniff a scent source, whether it is a novel or familiar scent), the discrimination ratio (novel entries divided by the total number of entries), and the time spent in the novel zone (s).

4.5. Depressive Behavior

We assessed depressive behavior using the tail suspension test (TST) and forced swim test (FST), two inescapable tasks that induce behavioral despair. For TST, mice were individually suspended at a height of 30 cm by attaching the tail to a horizontal ring stand bar using adhesive tape. Hollow tubes were placed around the tail to inhibit climbing tendencies. Each 6-min test session was videotaped laterally and scored (ANY-maze video-

tracking software v6.2) for escape-oriented behavior (mobility) and bouts of immobility. For the FST, mice were placed individually in a 4 L glass beaker with 30 cm of water maintained at 25 °C. Only the last 4 min of the 6-min session were videotaped from above and analyzed using ANY-maze video-tracking software. For both tests, the time spent immobile was recorded for each mouse as immobility correlates with depression-like behavior. According to ANY-maze, a mouse was deemed immobile when it was hanging/floating 65% passively for at least 2.5 s.

4.6. Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Following 3 mo of chronic Mn/V co-exposure, the mice were sacrificed in a CO₂ chamber, followed by cervical dislocation. Terminal blood was collected by cardiac puncture, and brain tissues were dissected, collected, and stored at −80 °C prior to analyses. Brain samples were analyzed for Mn and V concentrations using ICP-MS (Analytik Jena Inc. Woburn, MA, USA) in CRI mode with hydrogen as the skimmer gas. After thawing at room temperature, brain samples were processed and analyzed following the established SOP on a wet-weight basis. For quality control, bismuth (Bi), scandium (Sc), indium (In), lithium (Li), yttrium (Y), and terbium (Tb) were used as internal standards for the ICP-MS.

4.7. Data Analysis

Data analysis was performed using Prism 8.0 software (GraphPad, Boston, MA, USA). Behavioral raw data were analyzed using two-way ANOVA or mixed-effect analysis with Geisser–Greenhouse correction and Šidák’s multiple comparisons test. The brain’s metal concentration data were analyzed using a two-tailed unpaired t-test assuming a Gaussian distribution. All data are presented as mean ± SEM. Statistically significant differences are denoted as * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

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Data Availability Statement: The original contributions presented in this study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

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Conflicts of Interest: A.G.K. has an equity interest in Probiome Therapeutics located in Athens, GA. The terms of this arrangement have been reviewed and approved by the University of Georgia in accordance with their conflict-of-interest policies.

Abbreviations

The following abbreviations are used in this manuscript:

PD	Parkinson's Disease
Mn	Manganese
V	Vanadium
Tg	Transgenic
WT	Wild-type
α Syn	α -synuclein
ICP-MS	Inductively coupled plasma mass spectrometry
TST	Tail suspension test
FST	Forced swim test
BBB	Blood–brain barrier
CSF	Cerebrospinal fluid

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