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Dose-dependent response of metformin in enhancing motor performance and dopamine release in C57BL/6 mice afflicted by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

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ABSTRACT:

Introduction: *Clinically, metformin has been used as a cornerstone medicine in blood sugar homeostasis for further than 40 times It was obviously the first line treatment among type 2 diabetes mellitus (T2DM) cases. Recently, fresh places of metformin in cancer & neurodegenerative conditions came apparent. Then, we delved the capabilities of this magic medicine in enhancing motor performance, dopamine (DA) release and TH- protein expression.*

Methods: *C57BL/6 mice were grouped into 4 via; Group1 (Saline), Group2 (MPTP), Group3 (MPTP + Met200), Group4 (MPTP + Met400). After acute administration of MPTP (25mg/kg for 5- successive days) and attendant follow-up by metformin (200 & 400 mg/kg), mice were exposed to several behavioral tests and later sacrificed for amperometric DA release measures.*

Results: *MPTP mice showed a significant drop in motor functions and amperometric amplitude ($P<0.05$), as well as the vesicle recycling as measured by pair-pulse ratio. Interestingly, metformin proves decisive in mollifying the motor dysfunctions caused by MPTP, with Met400 being more potent. It inversely improves the DA release as well the expression of its biomarker (Tyrosine Hydroxylase) in both striatum and Substantia Nigra pars compacta. This, in substance, has always indicated a functional part of metformin in employing the motor functions and DA release in the Parkinson's disease (PD) mode.*

Conclusion: *Our study demonstrated that metformin enhances motor function, DA release, and DA expression in C57BL/6 exposed to acute MPTP-induced neurotoxicity, possibly through vesicle recycling. These findings may facilitate the clinical application of metformin in the treatment of motor and even non-motor symptoms of PD.*

Keywords: *Metformin; Parkinson's Disease; Dopamine Release; MPTP; Motor Performance.*

1. Introduction

Metformin is conventionally used as a foundation medicine in the treatment of type II diabetes mellitus (T2DM) and other metabolic syndromes (Patil et al. 2014). A long history of efficacy, energy, and safety has made metformin one of the most generally specified medications globally (Rena et al., 2013). Lately, it was set up to have profound eventuality in reducing the pitfalls of Parkinson's disease (PD) and other age-related central nervous system (CNS) diseases (Adedeji et al. 2014). The versatility of this magic medicine in upgrading the ruinous goods of both metabolic dislocations (e.g., T2DM) and neurodegenerative (e.g., PD) diseases is obviously due to the

participated dysregulated pathways (Santiago and Potashkin 2014). It's vehemently believed that exposure to environmental factors and inheritable vulnerability is markedly associated with the etiology and progression of both conditions (Bayliss et al. 2016). Also, a compelling number of attestations from epidemiological studies suggested that T2DM is a threat factor for PD, although an implicit link between PD and T2DM remains controversial (Chen and Tsai 2010). Metformin also promotes neurogenesis and enhances spatial memory conformation through the activation of atypical PKC-CBP pathway (Wang et al. 2012). Metformin, besides it is currently trending role in neurodegenerative diseases (Alzheimer's disease, AD, and PD), was also found to have a profound role in mitigating the pathogenesis associated with cancer, non-alcoholic fatty liver disease (NAFLD), inflammation, heart attack and polycystic ovarian syndrome (PCOS) (Mahmood et al. 2013). Also, it's an important seeker in suppressing appetite and promoting weight loss (Day et al. 2019).

Levodopa, the current gold-standard medicine in the treatment of motor symptoms associated with PD, has been under pitfalls due to its incapability to upgrade the ruinous non-motor symptoms. Still, under a long-term scale, it causes serious dyskinesia (involuntary muscle movements) called levodopa-induced dyskinesia (LID), and this, thus, corroborated the critical need for a more presumptive and potent medicine devoid of any long-term complications. Strong substantiation suggested the positivity associated with metformin in mitigating motor symptoms of PD and perfecting mitochondrial integrity by enhancing the upregulation of mitochondrial marker proteins such as heat shock protein 60 (HSP60) (Kang et al. 2017; Katila et al. 2017). Additionally, current progress is that mitochondrial dysfunction, oxidative stress (substantially caused by occupational exposures to environmental poisons), and protein mishandling have a crucial part in PD pathogenesis (Martin et al. 2011). The neuropathological hallmark of PD is the presence of alpha-synuclein eliminations called Lewy- bodies in the midbrain, associated with progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) (Kakish et al. 2015). However, the precise medium underpinning the part of Lewy- bodies in PD cases isn't well established. Whereas Lewy bodies may represent an original attempt to sequester misfolded proteins, they may, at some point, come as a trigger for a seditious response, which, in turn, damages DA neurons (Miller et al. 2019). In addition to Lewy bodies, activated microglia are also present in the brains of people with PD and in laboratory animals like mice and rodents. Inheritable factors are also attributed immensely to the etiology of PD, and until now, no available data has suggested the neuroprotective prowess of metformin on genetically caused PD. Numerous genes mutations similar to PINK1 (PARK6), LRRK-2, PARK2 (PRKN), MC-1, and UCHL-1 contributed putatively to the domestic PD (Lauretti et al. 2016; Pickrell et al. 2015). More so, gene mutations of PARK2 and PARK6 were reported to give rise to autosomal recessive youthful-onset Parkinsonism, a primary mitochondrial cytopathy (Goedert and Compston, 2018). However, it's imperative to understand that no available substantiation suggests that PD can be caused by inheritable factors single-handedly. However, a significant number of studies reported that a positive family history has been associated with a high risk of PD (Rotermund et al., 2018). Shen et al. (2018) reported that inheritable omission of vesicular glutamate transporter in DA neurons increases vulnerability to MPTP-induced neurotoxicity in mice.

In this study, we conducted four of the most constantly used behavioral assays (Open Field tests, Elevated Plus Maze, Rotarod, and Footprints), aimed primarily at the evaluation of motor activity, anxiety-related actions, and emotionality characteristics of C57BL/6 mice exposed to colorful treatments. We used an acute protocol of MPTP administration, which consists of administering 25mg/kg MPTP intraperitoneally for 5-successive days. As recently reported, metformin prevents dopaminergic neuron death in MPTP-induced mice models via autophagy and mitochondrial ROS concurrence (Patil et al. 2014; Martin et al. 2011). We recently found that metformin enhances DA release and, therefore, vesicle recycling in dopaminergic neurons. Although its role in PD has been reported, no previous study has delved into its dose-dependent role in perfecting DA release on MPTP mice models. Most of the studies conducted so far concentrated on assessing its part in enhancing the integrity of mitochondrial proteins and posterior scavenging of reactive oxygen species (ROS) (Kang et al. 2017). More so, the series of behavioral assays we conducted enabled proper evaluation of its role in motor performance and inescapably proved its eventuality in the treatment of motor symptoms.

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Positive results attained from open field test and elevated plus maze test further indicated its propensity in treating non-motor symptoms that are similar to anxiety, a hallmark that wasn't hypothecated ahead. Eventually, our study suggests that metformin is a neuroprotective agent that mediates motor function, DA release, and vesicle recycling, a role that might be of remedial value for PD cases. Metformin might also be a rising pharmacological seeker against neurological diseases and, particularly, as a future replacement for Levodopa.

2. Materials and Methods

2.1. Animals and MPTP & Metformin Treatments

Twenty-five male C57BL/6 mice weighing 25–30 grams have been used in this study. All the experiments were intuitively conducted in the central laboratory and animal center of the School of Life Science and Technology of Xi'an Jiaotong University (XJTU), China, under the guidelines and directives of the Ethical Committee for Animal Care Protocol and Use. Adequate measures were put in place in order to minimize animal death, pain, and/or discomfort. Mice were equally exposed to the trial/training phase prior to the behavioral tests for habituation and acclimatization.

The MPTP administration protocol was developed by Jackson-Lewis and Przedborski (2007) (with little modification). The acute protocol (25 mg/kg for five consecutive days intraperitoneally) was designed to produce desirable motor dysfunctions and dopaminergic loss. Since dopaminergic loss is a hallmark of PD, we analyzed whether acute MPTP administration can cause a loss of these DA neurons by investigating the level of tyrosine hydroxylase (being a major biomarker of PD) expression in both substantia nigra pars compacta (SNc) and striatum. Metformin (Sigma-Aldrich, ID# DST200524-178, CAS: 1115-70-4), on the other hand, was administered in two doses via mid-dose of 200 mg/kg and high-dose of 400 mg/kg. The potency and efficacy of each dose were analyzed accordingly.

Mice were kept for a couple of weeks in an animal center (five per cage) at light-dark cycles of 12 hours with free access to food and water. Prior to the treatments, mice were divided into four groups of 5-mice each and treated as follows: Group 1 was treated with saline and served as control, Group 2 was treated with MPTP (cumulative dose, 100 mg/kg of free MPTP), Group 3 was treated with MPTP and 200 mg/kg metformin (Met200) & Group 4 was treated with MPTP and 400 mg/kg metformin (Met400). After these treatments, mice were kept for a few days under the same environmental conditions and later sacrificed for further analysis using deep isoflurane anesthesia.

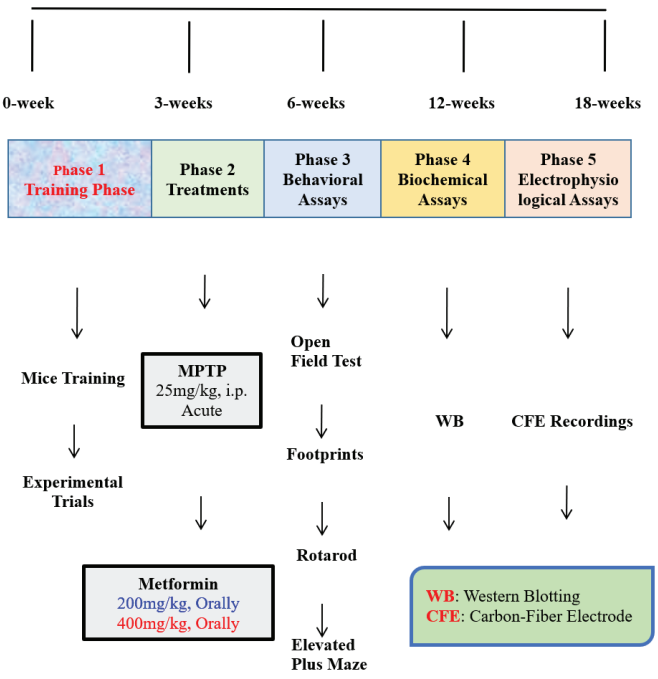

Figure 1 | Research Plan

The whole experiment was conducted in 3-4 months. 3-weeks are dedicated to mice's habituation and experimental trials. The next 3-weeks were dedicated to the administration of MPTP and Metformin. Four behavioral assays (EPM, OFT, Rotarod, and Footprints) were conducted between weeks 6th and 12th, including training. Mice were sacrificed, and biochemical and electrophysiological tests continued through week 18th.

2.2. Study Design

Thirty C57BL/6 male mice were randomly grouped into four as follows: Group 1 was treated with Saline and served as control. Group 2 was treated with MPTP (25 mg/kg intraperitoneally) for five consecutive days, and Group 3 and 4 were treated with 200 mg/kg and 400 mg/kg (met200 & met400) metformin, respectively. As an experiment that involved highly toxic

agents, five extra mice were added to each group as a backup. The mice were serially trained for 14 consecutive days (2 weeks) to acclimatize and adopt the different motor tests (**Figure 1**). Metformin (dissolved in Saline) was administered orally using a plastic enteral feeding tube. Behavioral assessment was carried out on the 4th day following the administration of MPTP and Metformin. Later, the mice were sacrificed, and brain slides were systematically cut for DA release measurement using carbon-fiber electrodes (CFE recordings).

2.3. Immunoblotting

Cells were isolated in lysis buffer containing 50mM HEPES, 150mM NaCl, 100mM NaF, 10mM sodium pyrophosphate, 5mM EDTA, 250mM sucrose, 1mM dithiothreitol and 1mM sodium orthovanadate with 1 % Triton-X and one tablet of complete Protease Inhibitor Cocktail (Roche, 11697498001) per 50mL, then stored at 80% until analysis. Protein concentration was determined with the Pierce BCA Protein Assay kit (Thermo Fisher). Lysates were then diluted with sample buffer and run on a polyacrylamide gel to separate proteins based on size. Next, samples were transferred to a polyvinylidene difluoride membrane and blocked in a 5% BSA for 1 hour at room temperature (20-25^{0}C). Membranes were incubated with primary antibody (1:1000 except α-actin 1:1,500) overnight at 4^{0}C. Appropriate secondary antibodies were used at a concentration of 1:10,000. Bound antibodies were detected using Clarity Western ECL Substrate (BioRad) (Vivacqua et al. 2020).

2.4. Open Field Test

In our laboratory settings, the Open Field Test (OFT) apparatus consists of a rectangular plastic arena measured (40 X 40 X 50) cm^3. The area is well-tight to prevent fluid absorption. The whole arrangement is connected with a video tracking system in which the area is divided into small squares of 5cm by 5cm. The trajectory path of the mice was traced and recorded within the center and corner zones, respectively, using an automatic video tracking system mounted above the maze. The open field maze was cleaned thoroughly after each experiment with 70% ethyl alcohol to get rid of the odor signal and allowed to dry completely prior to testing other mice. The mice were taken from their respective cages to the behavior room directly and tested once at a time for 30 minutes. Occasionally, a spherical beaker (or any other object) is placed at the center to measure the number of times the mice visited the region (center), and that will incontrovertibly mandate their social novelty, which, in substance, gives an evaluation of the position of depression and anxiety (Hwang et al. 2019).

2.5. Footprints

Footprint gaits were analyzed as preliminarily described by Wang et al. (2018). The hind paws and forepaws were coated with Red and Blue non-toxic ink. The mice were trained to walk along a 100cm-150cm long and 10cm wide open-top runaway (with 10cm high walls) with three runs per day for three successive days. A fresh sheet of white paper was placed on the bottom of the runway for each run. The footprint pattern was assessed quantitatively by stride length and front/hind footprint overlap or imbrication.

2.6. Rotarod

This test is used to evaluate mice's forelimb and hind-limb motor balance and coordination. In the 3rd week, mice were placed in a separate compartment on the rod and tested at an initial speed of 5 rpm until it reached 40 rpm. The latency to fall time (time on the rod) was recorded accordingly. Adapted from Ishaq et al. (2020) with little variations.

2.7. Elevated Plus Maze

The Elevated Plus Maze (EPM) test is used to assess anxiety-related behavior in mice models of Parkinson's disease and other Central Nervous System (CNS) disorders. The EPM set-up consists of a plus (+) – shaped maze elevated at roughly 100cm above the floor with two oppositely positioned closed arms, two oppositely positioned open arms, and a central area. As mice freely explore the maze, their behavior is recorded by means of a videotape camera placed above the maze and analyzed using a video tracking system. The preference for being in open arms over closed arms is calculated to measure anxiety-related behaviors. In order to detect zone entries and exits with perfection, the video was viewed in ANY-maze (Ojo et al. 2016).

2.8. Electrophysiological Recordings (DA

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Release Measurement)

Amperometric recordings in dorsal striatum slices were made using carbon fiber Electrodes (CFEs). Mice were anesthetized with isoflurane (1.5 g/kg, intraperitoneally) and transcardially perfused with approximately 50 ml ice-cold artificial cerebrospinal fluid-A (**Sectioning CSF**) (110 C5H14NClO, 2.5 KCl, 0.5 CaCl2, 7 MgCl2, 1.3 NaH2PO4, 25 NaCO3, 25 glucose mM, saturated with 95% Oxygen and 5% Carbon dioxide). Next, the brain was quickly removed and cut into 300-micrometer horizontal slices on a vibratome (Leica VT 1000; Nussloch, Germany). Slices containing the striatum were collected at +0.0 to 1.2mm from bregma. Slices were allowed to recover for 30 min in another artificial cerebrospinal Fluid-B (**recording CSF**) (125 NaCl, 2.5 KCl, 2 CaCl2, 1.3 MgCl2, 1.3 NaH2PO4, 25 NaCO3, 10 glucose mM, saturated with 95% oxygen and 5% Carbon dioxide at 37^{0}C, and then kept at room temperature for recording. CFEs 7 micrometers in diameter with an approximate 200-micrometer sensor tip were used to measure DA release in the striatum. The exposed CFE tip was fully fitted into the sub-surface of the striatal slice at an angle close to 30^0. A holding potential of 780 mV was applied to the electrode by an EPC9/2 amplifier and controlled by pulse software (HEKA Electronic, Lambrecht/Pfalz Germany). Single electrical field stimulation (Estim) pulses (0.2 ms, 0.6 mA) or trains (10 pulses at 20 Hz) were delivered through a bipolar platinum electrode (150-micrometer in diameter) and generated by a Grass S88K stimulator (Astro-Med). The amperometric current (I_{amp}) was low-pass filtered at 100 Hz and digitized at 3.13 kHz. The amplitude of amperometric current I_{amp} is proportional to the local DA overflow concentration [DA] with a calibration factor of 1 pA for nearly 6 nM. Off-line analysis was performed using Igor software (WaveMetrix). This protocol was adopted by Hwang et al. (2019) with little revision.

2.9. Statistical Analysis

Results are presented as the mean ± standard error of the mean (SEM). Statistical significance was assessed either via an unpaired 2-tailed student's t-test for two group comparisons or an ANOVA test with Turkey's HSD post hoc analysis for comparison of more than three groups. Statistical difference was considered significant at the level of $P < 0.05$ (5 % alpha). The results were analyzed using IgorPro, OriginPro2018, and SPSS 13.0 (statistical Packages for Social Sciences). Trajectory path/tract of the open field and elevated plus maze were obtained directly from the software (ANY-maze) and Bandicam independently with a videotape system mounted above the maze.

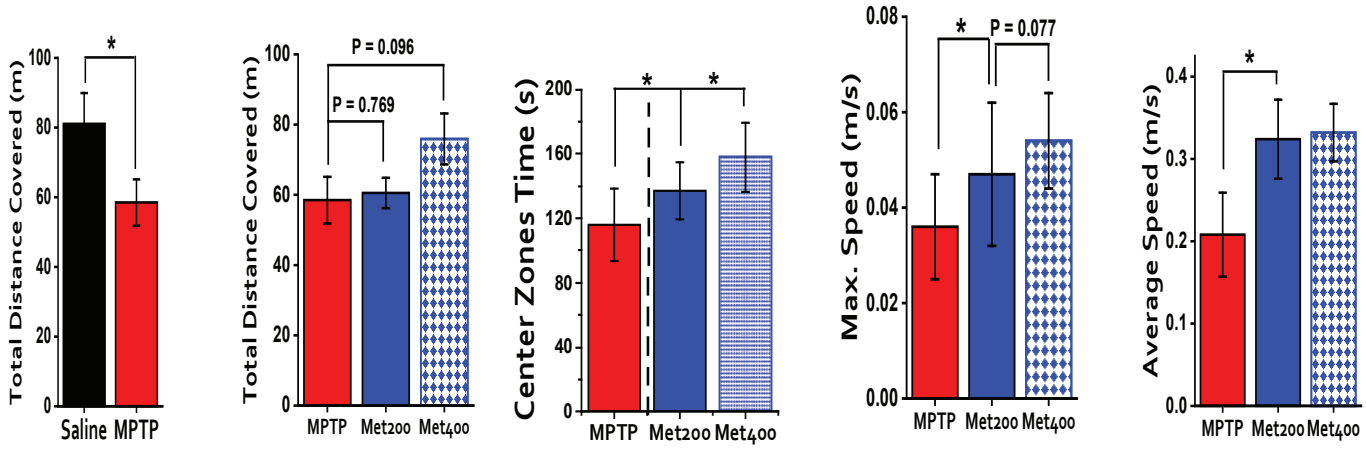
3. Results and Discussion

3.1. Metformin Improves locomotion in C57BL/6 mice

In this study, we conducted a series of behavioral tests using intact mice in order to evaluate the role of metformin in improving motor performance (especially locomotion). Open field test, being an important sensorimotor test used to determine gross locomotor activity and exploration habits in mice models exhibiting PD symptoms, was conducted in an open field maze with the mice allowed to move freely for 30 minutes while being recorded by an overhead camera. Total distance covered, time spent in pre-defined zones (center and corners), and average and maximum speed were all recorded and analyzed accordingly (**Fig. 2A-E**). The PD models (mice treated with MPTP) tend to cover a shorter distance and spent little time at the center of the maze when compared to the control (**Fig. 2A**). This, in essence, was attributed to the lack of balance and cognitive function following the predisposed DA neuronal loss in the striatum caused by MPTP. The cognitive dysfunctions (mostly anxiety and depression), however, are a result of precise damage of the DA neuron terminals in the ventral tegmental area (VTA) (Vivacqua et al. 2020). Another possible explanation for the decreased movement might be the increase in the actions of the direct pathway within the basal ganglia. It is obvious that deficiency of DA in the brain may lead to delayed and awkward movement (**Fig. F {PD models}**), and likewise, it is excess causes the body to make unnecessary movements such as repetitive tics or repetitious singularities (Ojo et al. 2016).

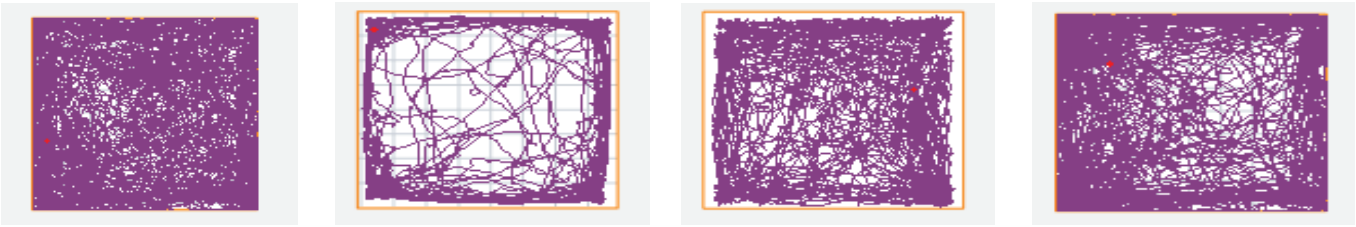
Metformin (especially Met400) showed some promising neuroprotective prowess with respect to locomotion as well as anxiety-related behaviors. The total distance covered by the MPTP group was improved handsomely following the administration of Met200 and Met400, with the latter being more potent and efficient (**Fig. 2A**). More so, the time spent in the inner/center zones in the MPTP group was improved significantly ($P < 0.05$) following the administration of

metformin. This indirectly measures thigmotaxis or wall-hugging behavior, and it is invariably indicative of anxiety-related behavior (**Fig. 2F**). Maximum and average speed further explained the redundancy of the mice to explore more areas of the maze, and this might be attributed to the depression they suffered following the MPTP damage (**Fig. 2D/E**). The overall result, in essence, proves beyond doubt that metformin has potential with respect to neuroprotection.



(A) **(B)** **(C)** **(D)** **(E)**

(F) Trajectory Paths as Recorded by Video Assistant System, VAS



Control **PD Model** **Met200** **Met400**

Figure 2 | Open Field Test

Saline, MPTP, Met200 & Met400 (n =5, each) mice were subjected to the OFT, and Total distance covered, time spent in center zones, and average and maximum speed were statistically analyzed. **Fig. 2A.** Bar chart representation of the total distance covered by saline/control and PD model/MPTP. **Fig. 2B**. Bar chart showing the total distance covered by MPTP and Metformin groups. Met200 covered almost the same distance as the MPTP group, whereas Met200 was higher but still statistically insignificant (P = 0.096). This showed some promising neuroprotective role of metformin in improving motor performance. **Fig. 2C**. Bar chart representation showing the time spent in the inner/center zones. Time spent in the center zones is very limited in the MPTP group when compared with metformin groups (P < 0.05). **Fig. 2D/E**. Bar charts represent the maximum and average as indicative of locomotion and redundancy. **Fig. 2F**. Trajectory paths as recorded by Video Assistant System, VAS: The mice were allowed to move freely in an open field rectangular space, and the corresponding movement (locomotion) was recorded by ANYMAZE software and video system. Center zones and corner zones were selected accordingly. MPTP-treated mice move more predominantly within the corner zones in contrast to the saline (control), which moves more evenly. The results for the data were expressed as mean ± SEM. Statistical analysis (*t-test*) was performed using SPSS and Microsoft Excel 2010. Differences were considered significant at $P < 0.05$.

3.2. Metformin Plays a Key Role in Anxiety-Related Disorders

Following the establishment of some non-motor function of metformin in C57BL/6 mice

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afflicted by MPTP, we investigated further using Elevated Plus Maze (EPM). EPM is used to assess anxiety-related behavior in rodent models of CNS disorders. As an anxiety test, we performed the test 3 days after the open field, Rotarod, and footprints in order to ensure full coordination and cognition. We analyzed their propensity to explore the open and closed arms of the maze, and from there, we evaluated the function of each treatment. In this study, we stationed open field tests together with EPM to evaluate spontaneous locomotors and exploratory conditioning. The number of arm entries (open and closed), as well as the duration spent in each, was the index we used to predict the anxiety-related behavior of each group. Interestingly, the time spent in the open and closed arms is significantly higher than that of the PD model (MPTP group) ($P < 0.05$). Metformin (Met400), on the other hand, increases the duration of the MPTP mice in both arms. The restoration conferred by Met400 was neither total nor absolute but proved decisive and promising. The explanation, then, is that when the mouse feels anxious, it tends to remain in the open arm, and that is what we discovered in our findings. The mice induced with MPTP (presumably, PD models) spent more time in the closed arms, and this clearly indicated an increase in anxiety as associated with PD subjects (**Fig. 3B**). We discovered that PD models have a limited number of arm entries and this indicated that, besides their motor impairment, there is a massive reduction in their cognitive function with respect to anxiety and depression (**Fig. 3C**). It appeared to us that the PD models feels very anxious to explore the various arms and slowly leads to slowness in decision and redundancy (**Fig. 3E**). We presumably postulated this theory based on the longest time they spent in the center when compared to other groups. Conclusively, motor impairment and cognitive function (specifically anxiety) are inversely proportional to the frequencies of arm entries and time spent in open arms. Furthermore, the impairment was to be directly proportional to the time spent in closed arms.

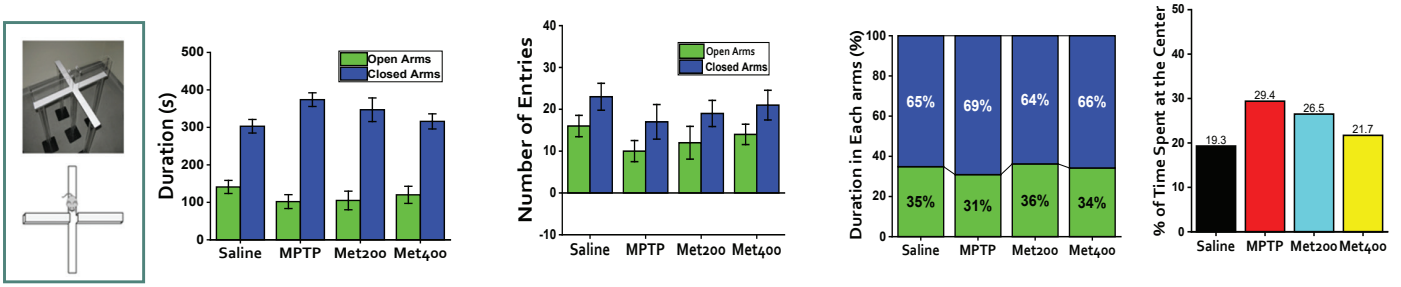

Figure 3 | Elevated Plus Maze Test

Fig. 3A. EPM set-up: The maze is settled with a height of about half-meter above the floor, with pillars standing from the ground floor. The maze is made up of two arms (2-open & 2-closed) crossing each other perpendicularly. The two paths facing each other have halls (hence called closed arms), and the other two have no walls (hence called open arms). **Fig. 3B.** Category Plot Bar chart representation of the time spent in the open and closed arms of the 4-groups. A very sharp and significant difference was observed between the two arms of each group ($P < 0.05$) and, obviously, higher time spent in closed arms. **Fig. 3C.** Bar chart representation showing the frequencies of arm entries. The frequency of arm entries decreases with an increase in motor impairment, and obviously, the MPTP group has the lowest. **Fig. 3D.** 100 % stacked column chart indicating the relative percentage of the time spent in each arm. **Fig. 3D.** A simple bar chart representation shows the percentage of time spent in neither of the arms (center) of the respective groups. MPTP group has the highest.

3.3. Metformin Enhances Balance and Motor Coordination

Motor coordination and balance were intimately evaluated and quantitatively assessed using the Rotarod test. The C57BL/6 mice were exposed to a series of experimental trials and 3-week training prior to taking readings/data to ensure full adaptation and learning the task to the same degree. Data were collected in batches throughout the 9th and 12th weeks (**Fig. E-G**). And at the end of week 12th (**Fig. B-D**). Testing consists of three sessions every week. Then, mice were tested on the accelerating rotation protocol in which they were placed on a rod that accelerates initially from 0-5

rpm and then gradually from 5 to 40 rpm until they collapsed. We observed carefully that the control and Met400 mice were able to withstand the rotating rod at the maximum speed (40 rpm) until they reached RAMP at 300 seconds (**Fig. 4B/D**). Meanwhile, the MPTP and Met200 mice withstood the rotation only hard and fell at a fairly lower time (**Fig. 4C**). As time progressed, we observed a massive improvement in the endurance, and metformin proved decisive over a period of 4 weeks. Initially (in the first 2 weeks), there was no statistical difference between the MPTP and metformin groups (Met200 & Met400). At week 3, we recorded a significant change in the endurance on the rod between the MPTP and Met400 (**Fig. 4G**), indicating positivity surrounding the neuroprotective training of metformin in improving motor performance; meanwhile, no progress was observed with respect to Met200. Unexpectedly enough, the loftiest latency to fall time was observed in the 3rd week, not 4th and the only conclusion we can decide then may be muscle fatigue, whereas the impotency in the first 2-weeks might be associated with motor learning skills/behaviors. Importantly, metformin showed some promising role in ameliorating the adverse effects of MPTP by nearly restoring balance and maybe social

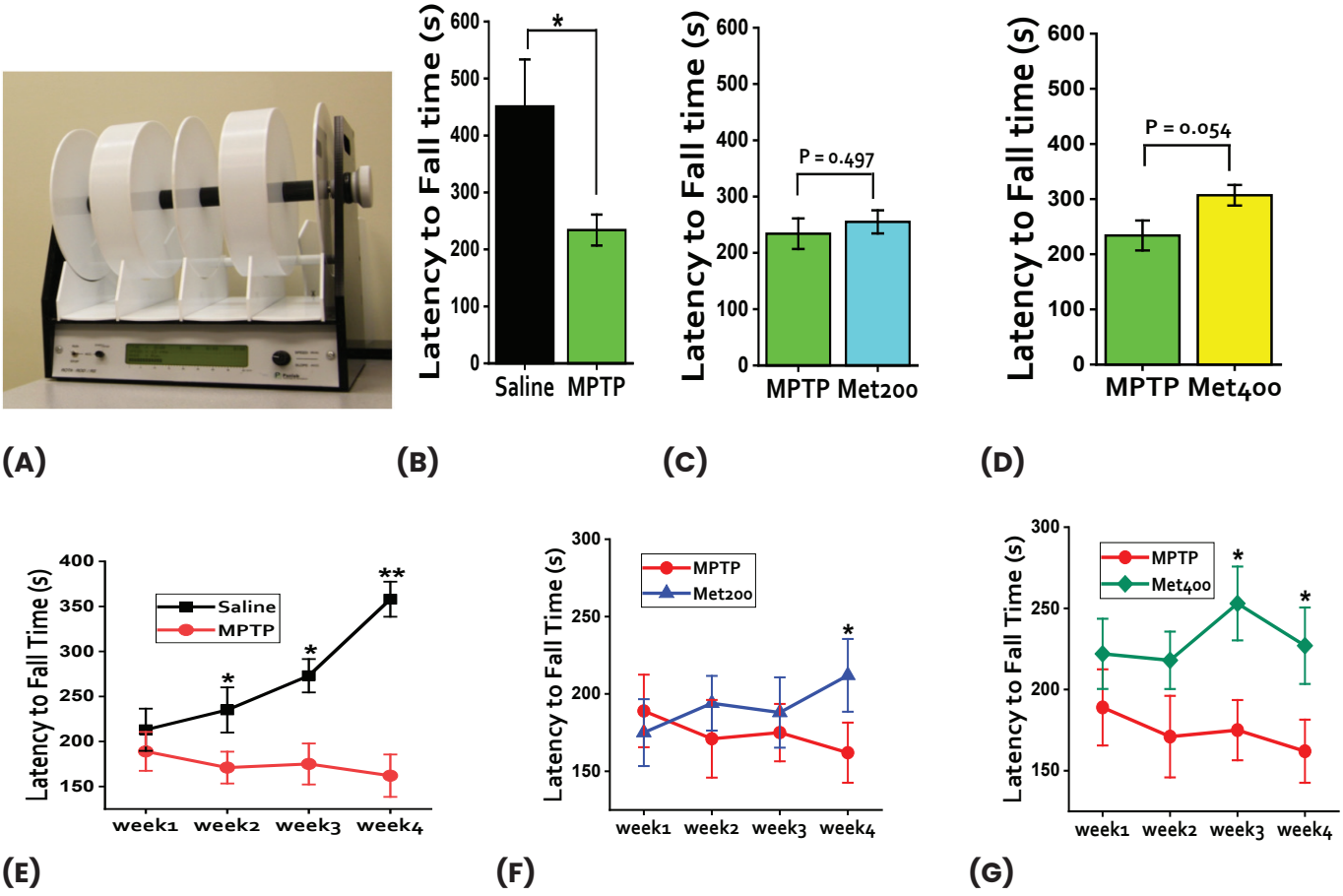

Figure 4 | Rotarod Test

Fig. 4A. Mouse Rotarod Set-up: The apparatus consists of a circular rod turning at increased speed (Initial of 5 rpm and Max. of 40 rpm). Mice were placed on the rotating rod to maintain balance and coordination. It is well automated, and we were able to test 5-mice at a time. Vertical metallic plates served as barriers to separate each. The latency to fall time was recorded and analyzed accordingly. **Fig 4B: Rotarod (Saline Vs. MPTP):** Expectedly, the Latency to falling time in the Saline is significantly longer than that of MPTP ($P < 0.05$). **Fig 4C. Rotarod (MPTP Vs. Met200):** Met200 failed **Fig. 4D. (MPTP Vs. Met400):** Increased time was sustained on the rod in the same group of mice treated with MPTP. Surprisingly, during the first 2-weeks, there was not much difference with the MPTP group. **Fig. E-G.** Analyze the endurance level in 4 weeks. Motor impairment increases with time as MPTP consistently causes damage, and subsequently, the gap widens (**E**). Met200 appeared to be less potent here, and the mice fell almost at the

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same time as that of the MPTP group (**F**). Values are expressed as mean ± SEM of the triplicate readings and considered significant at ** P < 0.05 and ** P < 0.01.*

3.4. Metformin Treatment maintained stride length in Footprint Gait analysis

It has been established profoundly that MPTP causes motor deficits such as involuntary movements (Di Biase et al. 2020). Objective evaluation and analysis of the gait cycle is, therefore, pivotal in understanding the severity and inflexibility of motor impairment. Gait impairment is an evolving condition, and a series of gait disturbances are evident as the disease progresses. Here, we assess the progression and severity of motor abnormalities induced by MPTP. Footprint gait analysis is used as an evaluation index. Metformin improves the unbalanced gait induced by MPTP (**Fig. 5C/E**). The stride length of saline, when compared to MPTP, was found to be significantly different at *P < 0.05*. They showed greater variation with the MPTP group in both the SD and range of stride length (**Fig. B/D**). Our findings proved that metformin can ameliorate the unsupportable deterioration caused by MPTP in the performance of motor tasks. Both Met200 and Met400 exert a very amicable impact in subsidizing deficits within cognitive and behavioral domains. However, in order to ascertain the impact, only 5-6 footprints of each group were sufficiently clear to be analyzed. Collectively, these findings indicate that metformin at both doses is sufficient to ameliorate locomotor deficits associated with gait abnormality, among others.

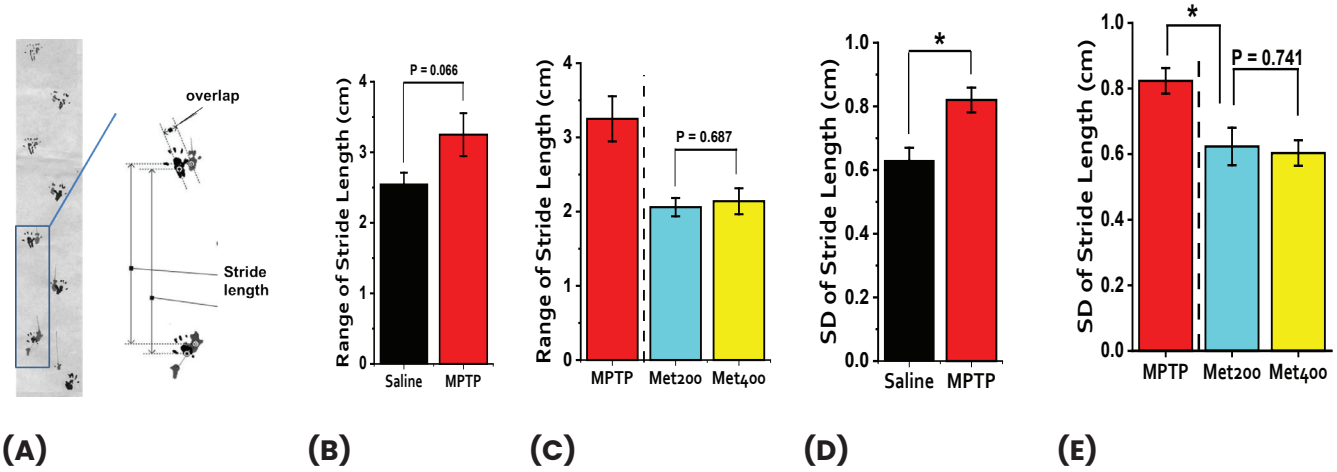

Figure 5 | Footprint Gait analysis

Fig. 5A. Footprints: footprints showing the stride length and overlap. **Fig. 5B.** Footprints of the MPTP and Saline. Concentrated stride length in the MPTP models indicates lower numbers of overlap as well as irregularity in their movement (gait abnormality). **Fig. 5C/E.** Footprints (MPTP VS Metformin). The number of overlaps indicates the regularity and order of movement. Expectedly, MPTP models showed a significantly limited number of overlaps (P < 0.001). Metformin reverses some of the motor deficits caused by MPTP (P < 0.01).

3.5. Metformin Improves DA Release and Vesicle Replenishment in both Striatum and SNc

Grounded on the compelling number of former research, we heavily believed and established the fact that MPTP causes a series of neurotoxic insults by inhibiting complex I protein of the electron transport chain (ETC) of the mitochondria (Hwang et al. 2019). This inhibition will integrate DA transporters (DAT) and block mitochondrial oxidative respiration, which will later result in the dysfunction of some mitochondrial proteins as well as the overall drop in mitochondrial energy and integrity (Mugikura et al. 2016). Accordingly, this will result in a lower release of neurotransmitters (specifically, DA) in the regions affected (Striatum and SNc). Then, we made amperometric recordings with electrochemical carbon-fiber electrodes (CFEs) in striatal and SNc slices to determine whether metformin can ameliorate the impaired DA release from the nigrostriatal terminals in mice induced by MPTP **(Fig. 6B)**. When a local original electrical stimulus (**Fig. 6A**) was applied to the brain slice (striatal slice in particular), there was a transient increase in amperometric current with a subsequent decay to baseline. This laterally represents a transient increase in extracellular DA concentration. Our findings

showed that metformin (especially Met400) can reverse some of the effects caused by MPTP (**Fig. 6C/G**). No former exploration or research delved into the neuroprotective role of different doses of metformin on MPT-induced PD models. Kang et al. (2017) conducted their evaluation on 6-OHDA PD models with no account of DA release. Next, we evaluated the role of metformin on vesicle recycling to determine whether it favors exocytosis and/or endocytosis by assaying the pair-pulse ratios at different stimulus intervals (10s, 20s, 30s, 40s) (**Fig. 6D/E/H/I**).

Expectedly, we discovered a massive reduction in the DA release and vesicle recycling in the MPTP mice as compared to the control. Both the amplitude as well as the pair-pulse ratios are reduced significantly ($P < 0.05$) in both the striatum and the SNc regions. And has inevitably indicated that vesicle replenishment was vehemently inhibited. However, burst stimulation using 10 a train of 10 pulses at a frequency of 20Hz revealed a reduced releasable vesicle pool in the striatal DA terminals only. Furthermore, we assessed the role of metformin in vesicle recycling by stimulating the regions at different times. Originally (at 10s and 20s), the DA release was veritably harmonious and steady but nearly the same as the baseline, and there was no significant difference between the groups. This, in essence, indicates that transient stimulation is sufficient to initiate release while potency/energy increases only with time (**Fig. 6D/H**). From the lower amplitude obtained in Met200, we stimulated only the Met400 strategically over four separate periods. Interestingly, we found that Met400 is potent enough to improve the DA release and vesicle recycling as time progresses (**Fig. 6E/I**).

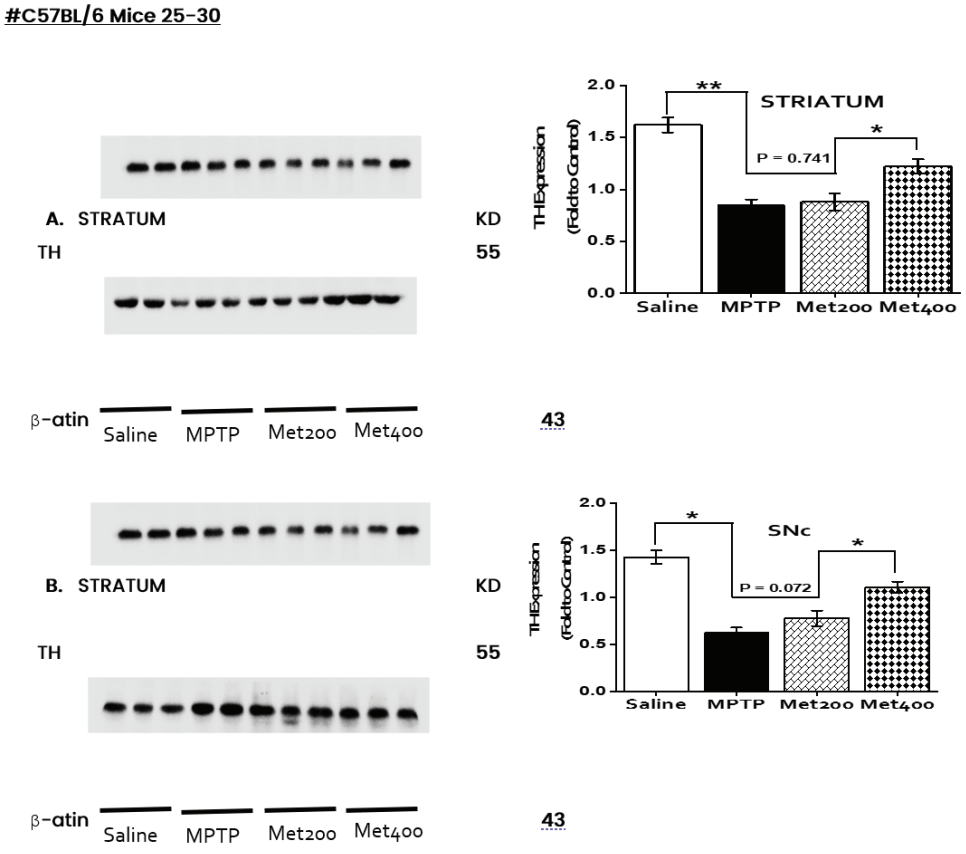

Figure 6A | DA release in Striatum and SNc

Fig. 6A: Carbon-Fiber Electrode Recording Set-up: Showing the arrangement for measuring the DA release in the striatum and substantia nigra. Representative amperometric currents in *Pico* ampere and statistics showing the intact DA release from dopaminergic terminals in the striatum and corresponding MPTP-damaged region. Carbon fiber is used to detect the release of DA. Estim serves as an electrophysiological stimulator that triggers the release of DA at different frequencies. **Fig. 6B/F: DA release of MPTP-mice:** striatal and SNc slices of the brain treated with MPTP show a massive reduction in the DA release when compared to the saline (P

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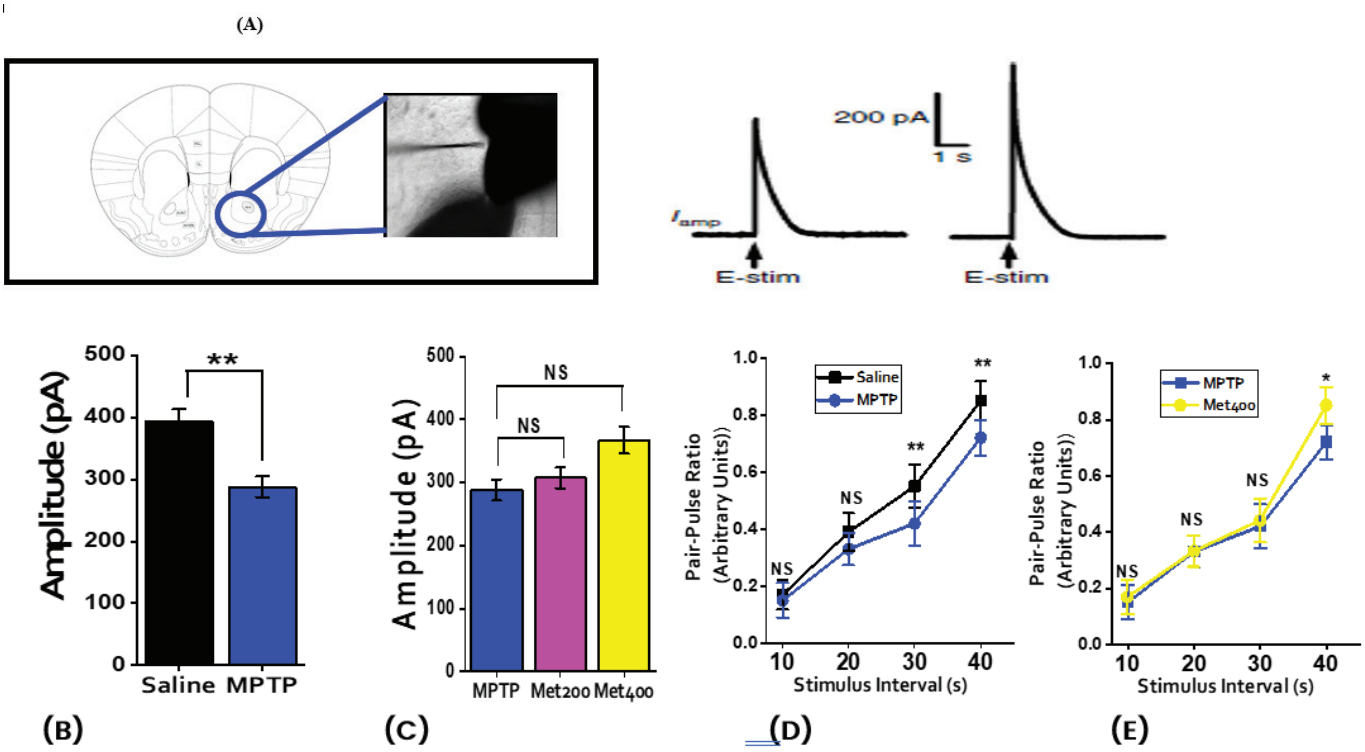
< 0.01) in both regions. This inarguably confirms the successful administration of MPTP to the target area and paves the way for further tests. **Fig. 6C/G: protective effects of metformin at different concentrations**: Both Met200 and Met400 slightly improve the release of DA in the striatum and SNc. Expectedly, the highest amplitude was detected at 400mg/kg in both regions. Metformin displayed a certain level of DA replenishment against the effect induced by MPTP significantly in the SNc (P < 0.01) and statistically insignificant in the striatum (NS, P = 0.939). **Fig. 6D/H: Vesicle Recycling (Saline vs. MPTP) in the striatum and SNc:** MPTP inhibits vesicle pool replenishment in dopaminergic neurons in both regions at the 30s and 40s stimulus intervals. **Fig. 6E/I: Vesicle Recycling (MPTP vs. Metformin) in the striatum and SNc:** Both Met200 and Met400 enhance the vesicle recycling in a stimulus-dependent pattern in the two brain regions. At 10s and 20s, the DA release is not significant enough as compared to baseline, and there is no improvement in the vesicle recycling, whereas, at 30s and 40s, there is enough recycling of dopaminergic neurons from their vesicles (P < 0.01) (P < 0.05) respectively. Pair-pulse ratios of DA release with different stimulus intervals (10s, 20s, 30s, 40s) were recorded accordingly. Data were collected from three different experiments and were expressed as mean ± SEM and unpaired student *t*-test.

3.6. Metformin enhances the expression of DA Biomarker (Tyrosine Hydroxylase) in both Striatum and SNc

Western blot analysis of the striatum and SNc of the MPTP mouse models showed that metformin (both Met200 and Met400) increased the levels of TH expression, and this may potentially lead to the defensive effects of metformin in rescuing dopaminergic neurons (**Fig. 7A/B**). Subsequent MPTP intoxication led to relative suppression of the catecholaminergic protein, TH, in both regions of the brain, and hence, metformin can only reverse the degeneration slightly. Based on our data, we found that the intoxication caused by MPTP was not unrecoverable, and the architecture of the affected regions can get back to normalcy with appropriate interventions. Interestingly, the striatum and the SNc of the group treated with metformin (especially Met400) demonstrated maintenance of TH expression when compared to the treated group (control). In conclusion, our results from this analysis suggested that metformin plays a crucial part in the survival of dopaminergic neurons in the midst of intoxication and subsequent oxidative stress. More so, a compelling number of studies have reported unequaled neuroprotective role(s) of metformin in MPTP-induced PD models. However, Curry et al. (2018) claimed that metformin is ineffective in 6-hydroxydopamine (6-OHDA)-induced PD models. Presently, no available data shows the neuroprotective prowess of metformin on rotenone-induced models.



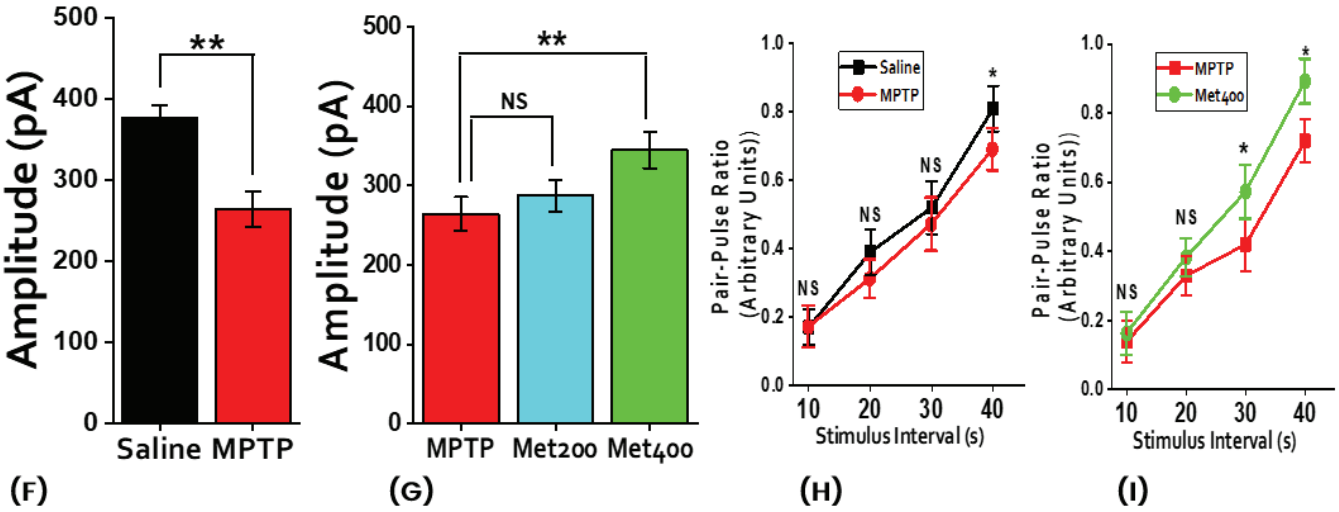


Figure 7 | TH expression in Striatum and SNc/Densitometric Analysis

Acute administration of MPTP decreases TH protein level. **Fig. 7A. Striatum:** Representative western blots showing the expression of Tyrosine Hydroxylase (TH) in the SNc and striatum of C57BL/6 mice treated with MPTP or metformin or both as determined by western blot with the indicated antibody. Western blots were performed 4 weeks after the last MPTP injection. **Fig. 7B.** Bar chart representation showing the optical density of TH protein expression in the striatum region of the brain. The expression in the MPTP-treated mice is significantly reduced when compared to control/saline ($P < 0.01$) and almost significant when compared to a lower dose of metformin (Met200) ($P = 0.741$). However, Met400 enhances the clarity and, hence, the expression of the TH-protein in the MPTP-treated mice significantly ($P < 0.05$). **Fig. 7C. SNc:** Representative western blots showing the expression of TH in SNc of the midbrain performed 4 weeks after the last MPTP injection. **Fig. 7D.** Bar chart showing optical density fibers in the SNc. A significant reduction in expression of the TH in the MPTP group was observed when compared to the control ($P < 0.05$), whereas Met400 rescued some dopaminergic neurons in the region ($P < 0.05$) as Met200 showed no difference. Values were expressed as fold to control and analyzed using Image J and OriginPro18 accordingly. * indicated a significant difference at 5% confidence interval (CI), ** at 1% confidence interval (CI).

3. Discussion

Metformin was well known for its role in metabolic syndromes and established a reputable status as a cornerstone remedial and therapeutic option for T2DM almost 5 decades ago. Recently, besides the aforementioned role, metformin was found to be effective in neurodegenerative diseases (e.g., PD) and cancer (Rotermund et al. 2018). Mechanistically, it affects several tissues that are central to metabolic homeostasis, including the pancreas, liver, brain, and skeletal muscle. In view of it is different physiological functions, it is not even surprising that metformin has an eventuality of improving motor deficits and DA release (Tayara et al. 2018; Lee et al. 2020)

In recent years, metformin was set up to be effective in perfecting motor symptoms of PD in experimental animals and also eased the L-DOPA-induced motor complications (Ryu et al. 2018). Motor dysfunctions similar to resting tremors, rigidity, and bradykinesia were the most common and indeed the most visible symptoms of PD and have a negative impact on the quality of life of cases with the complaint (Murata et al. 2016; Ishaq et al. 2020). So far, the open Field Test (OFT) is the most effective canonical assay for the relative assessment of changes in locomotor function (Samson et al. 2015). Regardless of the dose, metformin-treated mice showed increased entries of the center zone in the open field maze, which always indicated an increased curiosity and lower situations of anxiety (El-Sisi et al. 2015). This, thus, verified the role of metformin in ameliorating motor deficits. OFT, in combination with the Elevated Plus Maze test (EPM), is typically used to probe the behavioral function of certain compounds (e.g., anxiolytic agents) in preclinical settings involving PD models (Ojo et al. 2016; Ramos et al.

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2008; Sestakova et al. 2013). Here, we combined OFT and EPM results as indices of locomotion, anxiety, and exploration to predict the possible pharmacological role of metformin (Met400 in particular) in alleviating motor deficits caused by MPTP. As these two tests (OFT and EPM) all rely on the unconditioned avoidance of threatening situations and free moving within the maze, it could be hypothesized that metformin is not only effective in motor functions but also enhances non-motor deficits such as anxiety. In essence, Met400 improves locomotor function and mediates anxiety-related behaviors and gross motor functions in C57BL/6 mice induced by MPTP (**Fig. 2B-F & 3B-D**).

Having established the dose-dependent response of metformin on MPTP-induced PD models in locomotion and anxiety, we also set to explore the part of metformin in the conservation of balance and coordination. Postural balance, gait normality, and muscle strength were analyzed sequentially using Rotarod tests and footprints gait analysis (Hu et al. 2018). Here, we observed that mice treated with saline and metformin showed better balance and muscle coordination in the rotarod and footprints tests than the MPTP group (presumably PD). Groups treated with Met200 and Met400 may protect the dopaminergic neurons within the striatum from degeneration, leading to enhanced motor coordination, balance, and muscle strength. Also, as a hallmark of PD-like pathology in experimental animals, we measured the level of DA release using an electrophysiological carbon-fiber electrode. The amperometric amplitude (PA) was assessed, as well as the pair-pulse ratios at different stimulation intervals (Mosharov et al. 2005; Lahiri and Bevan 2020; Ivanova et al. 2020). Our findings, for the first time, reveal that metformin enhances DA release in a dose-dependent manner. Met400 was found to favorably increase the DA release magnitude compared to the MPTP group (**Fig. 6**).

Lower level and/or expression of TH- protein was one of the hallmarks attributed to PD pathogenesis. The expression of this biomarker is constitutively associated with catecholamines such as DA. Formerly, metformin was proved decisive in improving the number of TH-positive neurons in the striatum and SNc as evidenced by immunohistochemical assays/images (Patil et al. 2014; Bayliss et al. 2016; Kang et al. 2017; Vivacqua et al. 2020; Lee et al. 2020). Here, we observed that metformin has the ability to stimulate TH-protein expressions in both striatum and SNc. Met200 did not show any appreciable change compared to the control (**Fig 7**), and this demonstrated that only a high dose (Met400) of metformin is capable of inducing TH expression in both regions. A novel observation from our study is that the expression of TH in both striatum and SNc is fairly the same despite the unequal distribution of proteins.

A limitation of our study was that we could not give long-term experimental follow-up of metformin and the effects of metabolic phenotypes of C57BL/6 mice on the dose-dependent neuroprotective response of metformin and consequent deceleration of body weight in MPTP-treated groups. Therefore, further studies should concentrate on histological morphologies, body weight variation, long-term effects of metformin (say 3-4 months), and proteomic changes associated with the major mitochondrial protein biomarkers. This is in view of the fact that MPTP specifically inhibits complex I of the electron transport chain (Kang et al. 2017; Jackson-Lewis and Przedborki 2007; Lee et al. 2020; Williams-Langson et al. 1985) and inevitably disrupts the integrity of Mitochondria as a whole. The consequences of this might lead to metabolic changes, including weight loss.

4. Conclusion

Our study demonstrated that metformin enhances motor function, DA release, and DA expression in C57BL/6 exposed to acute MPTP-induced neurotoxicity, possibly through vesicle recycling. Our findings may facilitate the clinical application of metformin in the treatment of motor and even non-motor symptoms of PD. We greatly believed that metformin might be the future clinical substitute for Levodopa.

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Author Contributions

Daha Umar Ishaq: Conceptualization, Methodology, Software. **Daha Umar Ishaq.:** Data curation, Writing-Original draft preparation. **Daha Umar Ishaq**: Visualization, Investigation. **Daha Umar Ishaq**: Software, Validation: **Binta Garba Kurfi** and **Solomon Ojodemi Oguche:** Writing-Reviewing and Editing. **Daha Umar Ishaq** and **Solomon Ojodemi Oguche**.: Formal Analysis & Project Administration.

Disclosure Statement

The authors declared no potential conflicts of interests with respect to the research, authorship, and/or publication of this article.

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