Cannabinoid Receptor Modulation in the 3-Acetylpyridine Cerebellar Ataxia Mouse Model

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Research Article

Keywords: Cannabinoid, CB1 receptor, Purkinje cell, Cerebellar ataxia

Posted Date: January 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1198409/v1

Abstract

Cerebellar ataxia is a neurodegenerative disorder leading to severe motor incoordination. Recently it has been suggested that cannabinoids play a role in modulation of ataxic symptoms. In order to understand the possible therapeutic effect of cannabinoids for management of cerebellar ataxia, we used cannabinoid agonist/antagonists to target the cannabinoid type 1 receptor (CB1R) in the 3 acetyl pyridine (3AP) mouse model of ataxia. The role of the CB1R was examined by using three different doses of the CB1R agonist, WIN55,212-2 (WIN; 0.1, 0.5, 1 mg/kg) administrated 30 min prior to 3AP (55 mg/kg, i.p.) which leads to motor impairment through destruction of the inferior olive. In some recordings, the CB1R antagonist AM251(1 mg/kg) was given in combination with WIN. Locomotor activity and motor coordination were impaired by 3AP, and the application of WIN did not ameliorate this effect. However, the abnormal gait, rearing and grooming caused by 3AP were prevented by co-administration of AM251 with WIN. While the addition of the CB1R antagonist inhibition improved some ataxic symptoms, there was no effect of AM251 on balance or locomotor activity when co-administrated with WIN. Behavioral testing indicated that not only did WIN fail to exert any protective effect on ataxic symptoms, it exacerbated ataxic symptoms, suggesting that CB1R agonists may not be the ideal therapeutic drug in this disorder. When taken together, the findings from the present study indicate that cannabinoid modulation of ataxia symptoms may not act solely through CB1Rs and other cannabinoid receptors should be consider in future studies.

1. Introduction

Cannabinoid influences on the central nervous system (CNS) are varied and include disruption of the timing of cerebellar output, which influences motor learning. Alteration in timing of cerebellar output is relevant not only for motor learning, but also for chronic degenerative disorders involving dysfunctions in balance and motor coordination such as cerebellar ataxias. Since the discovery of the cannabinoid system, a wide range of neuroprotective properties of cannabinoids have been proposed for many neurodegenerative disorders, however, results exploring the role of cannabinoids in animal models of cerebellar ataxia remain controversial. Further, despite the widespread publicity about the medical benefits of cannabinoids, no clinical trials have been published on cannabinoids' role in ataxia, and only two case reports suggest that ataxia (with spasm) in MS may improve after inhaling cannabis or oral THC (Clifford, 1983; Consroe et al., 1997).

Cannabinoids act at two cannabinoid receptors, CB1R and CB2R. The CB1R is expressed predominantly in the CNS and its density is particularly high in the cerebellum, which suggests that cerebellum function is strongly regulated by CB1Rs. In support of this conclusion, CB1R activation has been found to modulate synaptic transmission at a few excitatory and inhibitory synapses of the Purkinje cells. Further, pharmacological studies have shown that activation of cerebellar CB1Rs is associated with symptoms of ataxia which has formed the basis for recommendations that blocking these receptors may reduce the symptoms of cerebellar ataxia (DeSanty and Dar, 2001; Patel and Hillard, 2001). However, some studies have lead to the suggestion that enhancing CB1R activity would be effective in management of ataxic

symptoms. Findings of deficient CB1R signaling in the cerebellar cortex in the du2J ataxia model indicated that enhancing CB1R signaling would improve ataxic symptoms (Wang et al., 2013). While conflicting, observations leading to these suggestions confirm that activation/inhibition of CB1 receptors could be useful for the development of therapies for ataxia (Fernández-Ruiz et al., 2015; Rodriguez-Cueto et al., 2016). However, it is still unclear whether agonism or antagonism of the CB1R receptor represents the best approach. Therefore, given the high-density of CB1R on the axon terminals of Purkinje cells that suggests CB1R involvement in cerebellar functioning and unclarity regarding whether agonism or antagonism of the CB1R is indicated in reducing ataxic behaviors, the aim of the present study was to explore the role of the CB1R on ataxia symptoms and the outcome of CB1R mediated modulation on these symptoms.

2. Material And Methods

2.1 Subjects and housing

A total of 96 adult male Wistar rats ranging in age from 4 to 6 weeks old were used. Rats were housed with free access to food and water under a 12 h light/dark cycle. The Institutional Animal Care and Use Committee of the Kerman Medical University approved all procedures, and the National Institutes of Health 'Guide for the Care and Use of Laboratory Animals' were followed. The animals were randomly divided into 12 groups (n = 8, in each group): Control, Saline + DMSO, 3AP, WIN-55,212-2 (WIN) (0.1, 0.5, 1 mg/kg), AM251 (AM) (1mg/kg), 3AP+WIN (0.1, 0.5, 1 mg/kg) and 3AP+ AM (1mg/kg). WIN (cannabinoid agonist) and AM (cannabinoid antagonist) were dissolved in DMSO. WIN was administered in three different doses (0.1, 0.5, 1 ml/kg) 30 min prior to 3AP (55 mg/kg, i.p.) once daily for 3 days. In a group of animals, AM (1mg/kg) was also administered 30 min prior to 3AP for three consecutive days. In the Saline +DMSO group, animals received the treatment once daily for 3 days. In the 3AP groups, rats received (55 mg/kg) only on the first day. In the 3AP+WIN+AM group, the rats received co-administration of WIN (0.1, 0.5, 1 mg/kg) and AM 30 minutes before 3AP injection once daily for 3 days. Following completion of experiments, and 24 hours after the last treatment, the animals were deeply anaesthetized by CO2 gas, their brains were removed, and the vermis of the cerebellum was rapidly separated and brain samples (n=4) of each group were protected in %10 formalin for histological tests.

2.2 Behavioral assays

2.3 Open field test

Locomotor activity was measured by movement within an open field (90 90 30 cm) for 5 min. Animals were transported to the experimental room about 1h before any session started in order to allow acclimatization to the room. At the beginning of the test session, the rat was placed in the center of the testing field. The number of times the animal reared and groomed, the time spent in the center of the open field (s), the total distance moved (TDM, cm), the total duration of mobility (s), and velocity (cm/s) were recorded. The floor was cleaned between sessions. A video camera was mounted 1.5 m above the floor of the open-field arena in a position which allowed the entire arena to be within view. The entire 5-

min session was recorded on videotape and analyzed off line using Ethovision software (version 7.1, Noldus Information Technology, Ethovision, Wageningen, the Netherlands) (Shabani et al., 2012a).

2.4 Foot print

This test was used to evaluate differences in gait. To obtain footprints, the hind feet of the rats were coated with nontoxic paints. The animals were then allowed to move freely across the walkway of a white sheet. To characterize the walking pattern, stance width and the distance between left and right hind footprints were analyzed.

2.5 Grip-strength test

The grip-strength test was used to quantify skeletal muscular strength (Shabani et al., 2011). Each rat was suspended with both forepaws on a horizontal steel wire [80 cm long, diameter 7 mm]. The animal was held in a vertical position when its front paws were placed in contact with the wire. When the rat grasped the wire, it was released, and the latency to fall was recorded with a stopwatch. Rats were randomly tested, and each animal was given three trials with a 30 min rest interval.

2.6 Evaluation of motor coordination.

To evaluate balance deficits of rats, we used the rotarod test (Hugo Sachs Electronik, Germany), which measures balance, coordination, and motor control. The rotarod accelerated from the minimum speed of 10 rpms to the maximum speed of 60 rpm. Rats were given three trials using a maximum time of 300 s with a 30 min rest interval. The trial was terminated when rats fell from the apparatus or after a maximum of 5 min. Rats were scored with disturbances in coordination if they fell from the test apparatus within 5 min (Shabani et al., 2012b).

2.7 Histopathology analysis

Following fixation with 10% formalin, the vermis of the cerebellum was dissected and embedded with paraffin, serially sectioned (4 μm). The slides were dewaxed and boiled (in a 600 W microwave oven) at 120 °C for 10 minutes. The sliced samples were stained with hematoxylin-eosin (H&E) and observed with a bright field microscope (Olympus CX31) (Abbasloo et al., 2015). Neurons that had distinctive nuclei and cell bodies were counted.

2.8 Statistical analyses

For the statistical analysis, two-way analysis of variance (ANOVA) was used, followed by the post-hoc Tukey test for comparisons among groups. Student's t-test was used to compare Control and DMSO groups and since there was no different between these groups, we compared other groups to DMSO instead of Control group. Data are expressed as mean ±SEM (All row data are presented in an Excel file as a supplementary file). The minimum level of significance was $P < 0.05$.

3. Results

3.1 Open field testing revealed locomotor and exploratory deficits in ataxic rats

In the open field, ataxic rats treated with WIN alone (0.1, 0.5, 1 mg/kg) or co-treated with WIN (0.1, 0.5, 1 mg/kg) and AM (1 mg/kg) moved quickly to the periphery compared to DMSO groups. DMSO rats did not move immediately and took more than twice as long as CB1-targeted rats to move to the periphery (Fig. 1A and B, $P<0.01$). This difference was significant, and indicates an increase in anxiety levels in ataxic rats treated with cannabinoids. Ataxic rats treated with WIN alone or co-treated with WIN and AM showed significantly more mobility in the open field than DMSO groups (Fig. 1C). There were no significant differences between groups in velocity and TDM in the open field during the 5 min period (Fig. 1F and G).

The frequency of supported rearing in which the forepaws were propped against the wall was higher in the ataxic rats treated with WIN compared to the DMSO group. Although, 3AP+WIN+AM rats exhibited a significant increase in rearing activity compared to the DMSO group, 3AP+ AM exhibited significantly lower rearing when compared to that of the 3AP group (Fig. 1D). The number of grooming was significantly lower in the 3AP group compared to DMSO rats, whereas the 3AP+AM group demonstrated a greater frequency of grooming compared to 3AP animals. A grooming cycle in a normal rat consists of an ordered sequence of face washing, body washing and scratching of the shoulders, chest and hindquarters (Berridge, 1990). These results indicate that AM251 improved anxiety-related behavior ataxic rats that were unable to perform complete grooming cycles (Fig. 1E).

3.2 Footprint

Footprint's analysis confirmed the marked difference in gait between control and 3AP rats (Fig. 2). While Control rats walked in a straight line, the ataxic rats lost their ability to control the direction of their movements, which indicated an impairment of motor coordination of the limbs. DMSO animals also exhibited a regular stride, whereas, ataxic rats displayed a significantly decreased stride width. Interestingly, as the dose of WIN increased, abnormality of gait was intensified and the high dose of WIN resulted in motor coordination deficits through a significant increase in the hind paw stride width. 3AP+AM indicated significantly increase stride width compared to 3AP rats (Fig. 2A(. However, no abnormality was observed in left and right stride length among any groups (Fig. 2B and C).

3.3 Rotarod and Wire Grip test

Functional changes in motor coordination were evaluated through the rotarod test. The WIN alone (0.1, 0.5, 1 mg/kg) or co-treated of WIN (0.1, 0.5, 1 mg/kg) with AM (1 mg/kg) groups showed an approximately 2 to 3 times lower degree of locomotor activity on the rotarod when compared to that seen in the DMSO group (Fig. 3A). This reduction in motor activity was also evident in muscle strength evaluation of the WIN alone or co-treated of WIN with AM groups in the wire grip test compared to the DMSO group. 3AP+WIN+AM treated rats held the wire shorter than the DMSO group (Fig. 3B), suggesting

that the addition of AM to effects of WIN could not improve the lack of strength and uncoordinated movement in these groups.

3.4 Histology

We preformed H&E histological tests to evaluate neuronal morphology features to determine effects of treatment on 3AP degeneration when CB1R-acting agents were present (Fig.4). Necrotic cells death was observed in 3AP and CB1 receptor agonist/antagonist pre-treatment groups compared to DMSO rats (Fig.4B). The higher the dose of WIN pretreatment, the greater the distortion of the Purkinje cell membrane in 3AP and WIN groups (Fig.4D). This pattern was not followed by 3AP+WIN+AM group (WIN 0.5mg/kg), in which it partially reduced the degeneration of Purkinje cell induced by 3AP and WIN (Fig. 4C).

4. Discussion

Balance, coordination, and locomotor activity degrade in cerebellar ataxia (Bastian, 2006). Studies have indicated that the cannabinoid receptors, particularly the CB1R, could be an ideal target for restoring movement (Baul et al., 2019; Leija-Salazar et al., 2020), however, discrepancies in the literature precluded making conclusions whether targeting CB1R agonist or antagonism was the best approach (Stephens, 2016). Therefore, in the present work, we examined the potential of cannabinoids as therapeutics in an animal model of ataxia. The model of ataxia utilized was the 3AP model. 3AP impairs the electron transport chain in the inferior olive. The inferior olive is the sole target of climbing fiber afferents to the cerebellum and the inferior olive plays a crucial role in motor timing and learning (Smeets and Verbeek, 2016). Therefore, metabolic induced neurodegeneration in the inferior olive by 3AP would be expected to lead to expression of ataxic symptoms (Colin et al., 1980). Consistent with this, 3AP administration resulted in a strong impairment of locomotor activity and motor coordination. Our finding showed the application of the cannabinoid receptor agonist WIN did not have any effect on reducing motor impairment in 3AP ataxic rats.

Interestingly, the abnormal gait, rearing, and grooming induced by 3AP were improved by the cannabinoid CB1R antagonist AM251. Aside from abnormal motor ataxic symptoms, ataxic rats also showed other behavioral deficits, particularly anxiety-like behaviors. They demonstrated reduction in grooming and exploration in inner zone of the open field arena, which shows enhancement in anxiety. Our results are in line with Glynn et al. who indicated that ataxic animals exhibited reduced exploration and grooming, which are sing of anxiety (Glynn et al., 2005). The effects of AM251 on these 3AP-induced symptoms do suggest that CB1 receptor inhibition could improve anxiety-like behavior in ataxic rats. Considering the improvement of grooming, it can be concluded that the cannabinoid antagonist may be useful in reducing anxiety-like behavior; however, more studies should be conducted to evaluate this hypothesis. On the other hand, as AM251 was not effective in normalizing balance or locomotor activity when co-administrated with WIN, our data suggests that targeting CB1R antagonism is not likely to improve all ataxia symptoms.

The effect of CB1R agonist on ataxia symptoms in absence or presence of AM251 was examined in the 3AP model at three different doses (0.1, 0.5, 1mg/kg). Behavioral tests indicated that not only did WIN fail to reduce severity of ataxic symptoms, it made the symptoms worse in a dose-dependent manner. Further, WIN was associated with a greater degree of PC cell death upon histological analysis. When taken together, these data suggest that cannabinoid agonists may not be the ideal therapeutic drug to pharmacologically manage cerebellar ataxia. However, there was a clear protective effect of CB1R antagonism apparent on PC survival. At the lower dose of WIN (0.5 mg/kg) necrotic PCs were relatively protected by co-administration of AM251. Nevertheless, the protected effect was limited as when AM251 was applied with the higher doses of WIN (1 mg/kg), necrosis of PC was intense. This partial protection effect of CB1R antagonism can also be seen in behavioral tests as the rearing of ataxic animals treated with WIN (0.5 mg/kg) was reversed by AM251, but AM251 had no effect in this behavior in ataxic rats when treated with the higher dose of WIN (1 mg/kg).

These results show that pre-treatment by AM251 and suppression of CB1 receptors did not significantly changed the balance and motor coordination as assayed in our behavioral tests. There are possibilities for this results, first: the AM251 dose was not enough to make significant change in balance and movement coordination. Second, although histological tests in 3AP+WIN +AM (WIN 0.5mg/kg) showed that AM251 protect the PCs, it did not appear in behavioral tests. Third, the cannabinoids modulation is not solely acting through CB1Rs. Previous studies are in line with our findings suggesting that ataxia is not purely a CB1-dependent mechanism, and have proposed that CB2 receptors may play a role in cerebellar ataxia. For instance, Navarro et al. reported that microglial CB2 receptors were up-regulated in the cerebellum of patients with different autosomal dominant cerebellar ataxias (Navarro et al., 2016). Similar findings were seen in spinocerebellar ataxia patients who demonstrated elevated levels of CB2 receptors in Purkinje neurons as well as in glial elements in the granular layer and in the cerebellar white matter. From their findings, Gómez-Ruiz et al. suggested that activating CB2 receptors may serve as neuroprotective therapies (Gómez-Ruiz et al., 2019).

The present study collectively indicated that unlike the majority of previous study recommendations, the CB1 is not the predominant receptor involved in cannabinoid modulation in the cerebellum. The reasons for this discrepancy are not clear. However, since the role of the CB2 receptor is still controversial, further investigation in this regard is suggested.

Declarations

Conflict of interest

The authors declare no conflict of interest and declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Acknowledgments

The authors thank Dr. Moazameh Razavinasab for helping in the illustration provided in this Article.

Funding:

No Funding.

Ethics approval:

All procedures performed in studies were in accordance with the ethical standards of the ethical committee of Kerman University of Medical Sciences (Ethical approval number: IR.KMU.REC.1398.012, Reg. No. 97000860).

Consent to participate:

The protocol was established, according to the ethical guidelines of the Helsinki Declaration, and was approved by the Institutional Ethics Committee of of Kerman University of Medical Sciences.

Consent for publication:

Not applicable.

Conflict of Interest statement:

The authors declare that there are no conflicts of interest.

Data availability:

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Code availability:

All software applications used are included in this article.

Author contribution:

HR and MS have conceived and designed the concept and road map of the study, searched the literature, designed the concept map and figures, and drafted the manuscript. MJ and KAK have critically reviewed the manuscript for its content, originality, usage of English language, and accuracy of interpreted data. MSH designed the study, helped in manuscript preparation, and critically reviewed the manuscript. MSH is the archival author and attests to the integrity of the original data and the analysis reported in this manuscript. All authors have made substantive contribution and attest to approving the final manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

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Figures

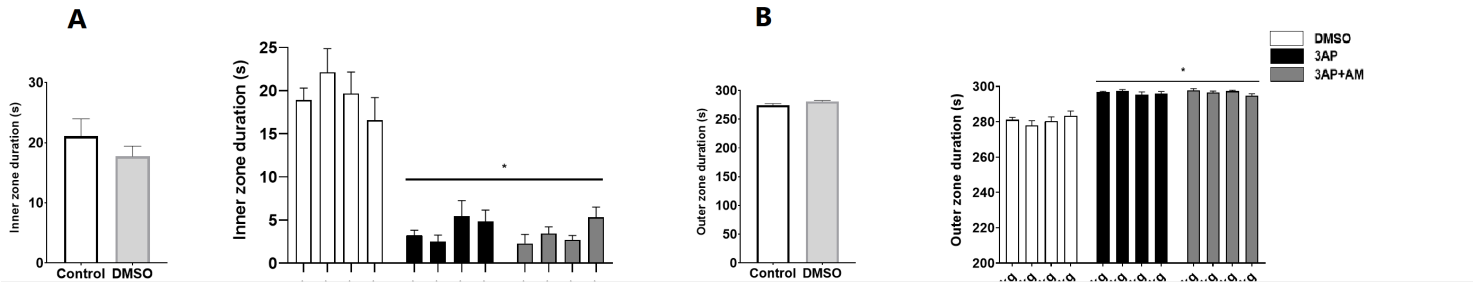

Figure 1

Characterization of animal's exploratory activity in the open field test. The time spent in A) inner and B) outer zones of the test arena, C) duration of immobility duration, D) frequency of rearing, E) number of grooming, F) speed of walking, G) total distance walked. Data are expressed as the mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001 compared to DMSO).

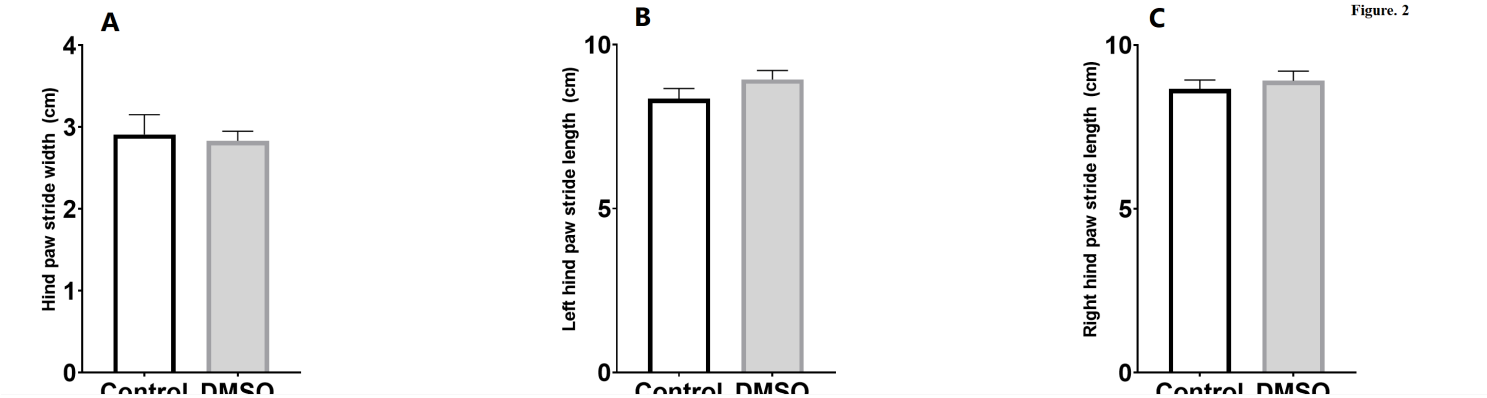

Figure 2

Gait analysis in the footprint test. (A) Stride length (cm), (B) left hind stride length (cm), and (C) right hind stride length (cm). Data are expressed as the mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001).

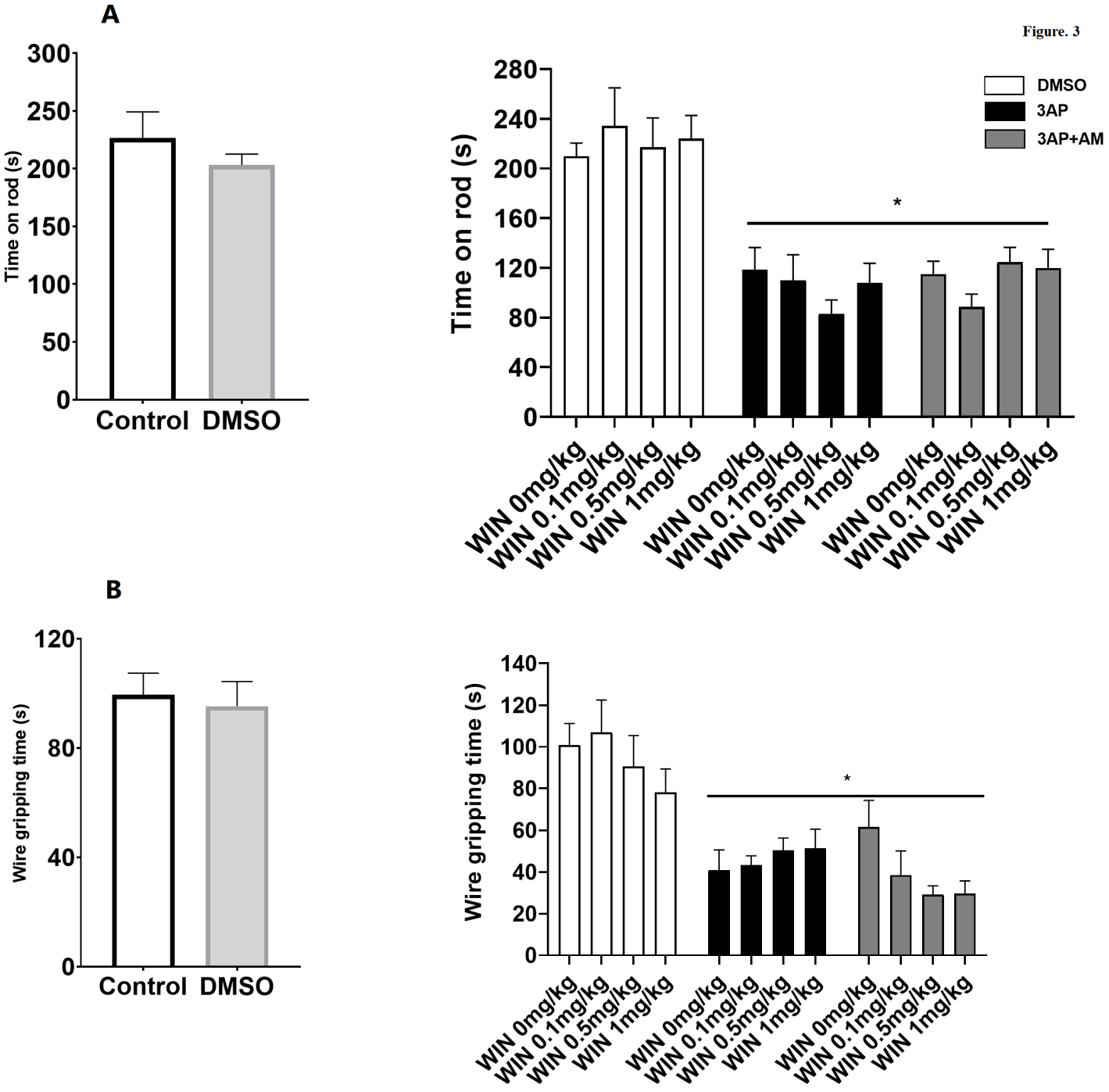

Figure 3

Rotarod and grip strength analysis. A) rotarod, B) Hang wire tests. Data are expressed as the mean ± SEM (* P < 0.05, ** P < 0.01, *** P < 0.001).

Figure 4

Purkinje cell degeneration was evaluated by H&E test. A) DMSO group, B) 3AP+WIN (1 mg/kg), C) 3AP+WIN +AM (WIN 0.5mg/kg), D) 3AP+WIN (1 mg/kg) +AM (WIN 1 mg/kg).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- WINAMHODA.xlsx