# Cannabinoid type 1 receptor antagonism ameliorates harmaline-induced essential tremor in rat

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**Abstract**

**Background and purpose:** Essential tremor (ET) is a neurological disorder with unknown etiology. Its symptoms include cerebellar motor disturbances, cognitive and personality changes, hearing and olfactory deficits. Excitotoxic cerebellar climbing fibre hyperactivity may underlie essential tremor and has been emulated in rodents by systemic harmaline administration. Cannabinoid receptor agonists can cause motor disturbances although there are also anecdotal reports of therapeutic benefits of cannabis in motor disorders. We set out to establish the effects of cannabinoid type 1 receptor agonism and antagonism in an established rodent model of ET using a battery of accepted behaviour assays in order to determine risk and therapeutic potential of endocannabinoid system modulation in ET.

**Experimental Approach:** The behavioural effects of systemic cannabinoid (CB) receptor agonist (0.1, 0.5 and 1mg $kg^{-1}$ WIN55, 212-2) and antagonist (1mg $kg^{-1}$ AM251 and 10mg $kg^{-1}$ rimonabant) treatment on harmaline-induced (30mg $kg^{-1}$) tremor in rats was assessed using tremor scoring, open field, rotarod, grip and gait tests.

**Key Results:** Overall, harmaline induced robust tremor that was typically worsened across the measured behavioural domains by CB type 1 ($CB_1$) receptor agonism but ameliorated by cannabinoid type 1 receptor antagonism.

**Conclusions and Implications**: These results provide the first evidence of effects of endocannabinoid system modulation on motor function in the harmaline model of essential tremor and suggest that $CB_1$ receptor manipulation warrants clinical investigation as a therapeutic approach to protection against behavioural disturbances associated with essential tremor.

| **TARGETS** | | **LIGANDS** | AM251 |
|---|---|---|---|
| **GPCRs** | $CB_1$ | Harmaline | Rimonabant |
| **Ionotropic receptors** | GlyR | WIN55,212-2 | |

**Abbreviations:** ET: essential tremor; PC: Purkinje cell; MS: multiple sclerosis;

**Introduction**:

Simple essential tremor (ET) is a neurological disorder of unknown etiology (prevalence: 0.4-3.9%), typically affecting upper limbs and, less commonly, the head, jaw, tongue, trunk and lower limbs. Although a syndrome of tremor in posture and movement, cerebellar motor disturbances, cognitive and personality changes, and hearing and olfactory deficits are also associated with ET (Deuschl *et al.*, 2009). Interest in essential tremor remains high due to its relatively high prevalence, adverse effect upon quality of life (Schmouth *et al.*, 2014) and apparently increasing prevalence in diseases like multiple sclerosis (MS) (~25%) (Fox *et al.*, 2004). ET treatment includes pharmacotherapy with beta adrenoceptor blockers, anticonvulsants, neuroleptics and antidepressants, although surgical treatments are required in the ~50% of cases that are pharmacoresistant (Chopra *et al.*, 2013) demonstrating a significant unmet clinical need (Koller *et al.*, 1989).

Excitotoxic climbing fibre hyperactivity has been suggested as one possible cause of ET and can be emulated in laboratory species by harmaline (i.p.), a beta carboline derivative of harmala alkaloids from *Peganum harmala* (Syrian Rue) seeds. Harmaline produces an 8–16 Hz tremor in mice and rats and, in rats, is associated with Purkinje cell (PC) loss (Handforth, 2012).

Recent studies have revealed a role for endocannabinoids in tremor disorders (Glass, 2001; Howard *et al.*, 2013; Arjmand et al., 2015). Cannabinoid (CB) receptors and their endogenous ligands, the endocannabinoids, are abundant in brain areas that manage motor function where they play a neuromodulatory role (Rodriguez de Fonseca *et al.*, 1998). Abundant cerebellar, CB type 1 ($CB_1$) receptors expression, particularly on PC inputs from interneurons and excitatory climbing fibres arising from granule cells and PC synapses emphasises the importance of endocannbinoid signaling in the cerebellum where it modulates classical cerebellar neurotransmission via activity-induced inhibition of presynaptic neurotransmitter release through $K^+$ channel-mediated inhibition of presynaptic $Ca^{2+}$ influx (Daniel *et al.*, 2004).

Although specific changes to cannabinergic signaling in motor diseases remains unclear and significant gaps in our understanding of cannabinergic influences on motor pathways remain, patients have claimed therapeutic benefits of medical cannabis in tremor-associated diseases (Clifford, 1983). Reduced tremor and spasticity in animal models of MS has been reported following treatment with □$^9$-tetrahydrocannabinol, a psychoactive plant cannabinoid (Koch *et al.*, 2007; Baker et al., 2000) and numerous but unsubstantiated patient claims for benefits of cannabis use in ET have been made (Tudge *et al.*, 2015). Interestingly, an extensive literature shows dose-dependent effects of the CB receptor partial agonist, □$^9$-tetrahydrocannabinol (THC) in this regard (Frederickson *et al.*, 1976; Kujtan *et al.*, 1983; Stanford *et al.*, 1998; Freedland *et al.*, 2002). Most notably, a systematic review revealed that $\Delta^9$-THC was probably ineffective for easing MS-related tremors (Koppel *et al.*, 2014) while, conversely, sustained use of $\Delta^9$-THC-rich extracts reduced tremor and spasticity in MS (Buccellato *et al.*, 2011). Thus, a confusing literature surrounds cannabinoid effects upon tremor in MS and, to date, no studies have investigated cannabinoid effects in ET, a discrete disorder. Therefore, here we report effects of a CB receptor agonist and $CB_1$ receptor antagonists on harmaline-induced tremor in rats, using behavioural measures to determine whether endocannabinoid modulation represents a plausible therapeutic strategy for the treatment of ET, in addition to assessing potential risks associated with therapeutic or recreational use of cannabinoid preparations by ET patients.

**Methods:**

Male Wistar Kyoto rats (40–60g (P24-28); Kerman Neuroscience Research Center) were used in accordance with National Institutes of Health guidance and approved the Kerman University of Medical Sciences. Information regarding the use of animals reported herein complies with ARRIVE guidelines (McGrath & Lilley, 2015). Animals were group housed (2-3 animals per cage) in conventional laboratory rodent cages (Razirad Co., Iran) of dimensions 26.5 (W) x 15 (H) x 42 (L) cm and maintained on a 12h light–dark cycle at a 23±2°C with access to food and water *ad libitum*. Experiments were conducted during the light phase (08:00-16:00h).

Three experiments (see **Experimental design** below) were undertaken, each of which employed five behavioural tasks: tremor scoring, open field test, rotarod test, grip strength test and gait analysis test (Vaziri *et al.*, 2015). Tests were administered sequentially. Pilot studies (n=16) revealed that 30mg $kg^{-1}$ harmaline induced stable tremor in this population for the duration of the testing period (2.5-3hrs). Previous studies have revealed that harmaline produces tremor at doses of 9-50mg $kg^{-1}$ in laboratory rodent species (Handforth, 2012).

**Behavioural assays:**

**Tremor scoring**

Tremor was rated by two observers blinded to treatment. Intra- and inter-observer reliability were assessed via kappa coefficient (acceptance criterion: >80%). Tremor data were acquired during the open field test and quantitatively scored as follows: 0: No tremor, 1: occasional tremor affecting only the head and neck, 2: intermittent (occasional tremor affecting all body parts), 3: persistent (persistent tremor affecting all body parts and tail), 4: severe (persistent tremor rendering the animal unable to stand and/or walk) (Al-Deeb *et al.*, 2002). Number of rearing events (standing on hind paws with a body-floor angle >45° (Lamprea *et al.*, 2008) (a measure of vertical and explorative activity related to locomotor behaviour) and number of grooming events (coordinated, patterned, obsessive motor action

(Komorowska *et al.*, 2004; Kalueff *et al.*, 2007) of front paws or mouth on the fur) per session were also recorded.

**Open-field test assessing locomotor behaviour**

A Plexiglas arena (90 [W] × 90 [L] × 30 [H] cm) was used. Each animal was placed in the center of the arena and horizontal activity recorded for 5 minutes with subsequent offline analysis (Ethovision 7.1, Noldus Information Technology, Netherland) that assessed total distance moved, duration of mobility and speed. The chamber was cleaned with 70% ethanol and dried between sessions (Vaziri *et al.*, 2015).

**Rotarod test**

Motor and balance performance were evaluated by accelerating rotarod device (Hugo Sachs, Germany). Prior to placing an animal on the apparatus, rod rotation was set to 10 rpm. At test start, the animal was placed on the rod which was linearly accelerated at 10 rpm/minute to a maximum of 60 rpm. Each animal undertook three trials with a 30 min inter-trial rest interval. The duration for which each animal remained in the apparatus was recorded and the mean for all trials per animal calculated (Vaziri *et al.*, 2015).

**Wire grip test**

The wire grip test assesses muscle strength and balance (Marks *et al.*, 2009). Each animal was suspended by both forepaws from a horizontal steel wire [80cm long, 7mm diameter] suspended 45cm from the ground. Each animal was held in a vertical position when its front paws were placed in contact with the wire. When the animal grasped the wire, it was released and latency to fall recorded with a stopwatch. Each animal undertook three trials with a 5 minute inter-trial rest interval.

**Gait analysis test**

The gait analysis test assesses animal walking patterns and gait kinematics. The hind paws of each animal were marked with a non-toxic ink and the animal allowed to traverse a clear Plexiglas tunnel (100 cm [L]×10 cm [H]×10 cm [W]) lined with white absorbent paper (100 cm × 10 cm) and ending in a darkened cage. The resulting tracks provide the spatial relationship of consecutive footfalls from which animal stride length and width were measured. Animals were habituated to the runway for 3 training runs before testing. Hind paw stride lengths were measured by distance (cm) between the respective paw prints to the successive ipsilateral prints to assess uni- or bi-lateral effects of treatment upon gait. Hind paw stride widths were measured by distance between the centers of the respective paw prints to the corresponding contralateral stride length measurements at a right angle. Footprints at the beginning and end of each run were not considered in the analysis (Wecker *et al.*, 2013).

**Drugs**

The non-selective CB receptor agonist, WIN55, 212-2 (Sigma, USA), and $CB_1$ receptor selective antagonists, AM251 (Sigma) and rimonabant (Cayman, USA) were first dissolved in dimethylsulfoxide before further dilution in $dH_2O$ (DMSO; maximum DMSO concentration: $1\%^{v}/_{v.}$ Harmaline hydrochloride dihydrate (Sigma) was dissolved in $dH_2O$. Drugs were administered i.p. to a maximum total injection volume of 1 ml.

**Experimental design**

The present study comprised three discrete experiments. **Experiment 1** assessed the effects of harmaline in the behavioural tests described. Here, two groups of animals were employed, one of which received harmaline (30 mg $kg^{-1}$; i.p.) and the other harmaline vehicle ($dH_2O$; i.p.), each 15 minutes before behavioural testing began. **Experiment 2** assessed the effect of CB receptor agonism upon harmaline-induced symptoms. Here, four groups of animals were used where one received WIN55, 212-2 vehicle (i.p.; administered 30 minutes

before harmaline) plus harmaline (30 mg $kg^{-1}$; i.p.; 15 minutes before behavioural testing) and three received WIN55, 212-2 at doses of 0.1, 0.5 & 1 mg $kg^{-1}$ (i.p.; administered 30 minutes before harmaline) plus harmaline (30mg $kg^{-1}$; i.p.; 15 minutes before behavioral testing). Finally, **Experiment 3** examined the effects of $CB_1$ receptor antagonism upon harmaline-induced symptoms. Here, three groups of animals were used where one received AM251/rimonabant vehicle (i.p.; administered 30 minutes before harmaline) plus harmaline (30mg $kg^{-1}$; i.p.; 15 minutes before behavioral testing) and two received either AM251 (1mg $kg^{-1}$; i.p.; administered 30 minutes before harmaline) or rimonabant (10mg $kg^{-1}$; i.p.; administered 30 minutes before harmaline) plus harmaline (30mg $kg^{-1}$; i.p.; 15 minutes before behavioral testing).

*In vitro*, AM251 exhibits greater affinity for $CB_1$ receptors (3-10 fold; dependent on assay) and exerts greater inhibition of agonist effects at $CB_1$ receptors (6-10 fold difference in $IC_{50}$; dependent on assay and agonist) (Pertwee (2005). Therefore, AM251 and rimonabant were employed at doses of 1 and 10 mg $kg^{-1}$ respectively. CB receptor agonists and antagonists employed in the present study were also examined for effects in the tasks described when administered in the absence of harmaline (doses as stated above; i.p.; 45 minutes before testing began; See ***Supplemental Results***; Figures S3A & S4A vs other supplemental Figures S1-S4). Briefly, when administered in the absence of harmaline, only rimonabant treatment affected any measure where a decrease in rearing events and time on rotarod were observed.

**Statistical analysis**

On entry into the study, 192 animals were randomized, using an online tool (http://graphpad.com/quickcalcs/randomize1/; seeded using the time of day) into 16 groups of 12 animals as described. Where animals failed to complete a task and provide valid data, no value was included for analysis. Reasons for task failure included: failure to habituate to handling, failure to habituate to equipment, technical (e.g. equipment) failure or data provided not amenable to robust analysis (e.g. indistinguishable footprints in the gait task).

The number of animals per group per assay that contributed data for quantitative analysis are shown in parentheses in each figure. Group size was determined by sample size calculation to provide statistical power of ≥80% to detect effect sizes consistent with relevant comparators previously described for this animal model (Handforth, 2012) at the 5% level of significance with the intention to establish differences between control and study drug groups.

Experimental data were collected by researchers blinded to drug treatment and analysed by an independent researcher blinded to group identity. Data were unblinded prior to pairwise statistical comparisons (see below) in order to allow specification of the comparator control group. SPSS (IBM, USA), Origin (OriginLab Co., MA, USA) and GraphPad Prism 6 (GraphPad Software, USA) were used for statistical analysis of data and figure production. Prior to the conduct of comparative statistics, the presence or not of outlier data points pooled by task was assessed using the ROUT method as implemented in GraphPad Prism 6 (Motulsky & Brown, 2006). These data were excluded from the statistical analysis and comprised 6/1648 (~0.3%) data points across all groups and all assays. Data were then assessed for normality using a Kolmogorov-Smirnov test. Results found to be normally distributed ($p>0.05$ in K-S test) were expressed as mean±SEM and analyzed using either a paired Student's t-test or a one-way ANOVA test. Where a main effect was seen in ANOVA tests, pairwise comparisons between control and each drug treated group were then made using Tukey's post-hoc tests. Results that were not normally distributed ($p<0.05$ in K-S test) were expressed as median and interquartile range (expressed as median (interquartile range)) and analysed using either a Mann Whitney test or a Kruskal-Wallis test. Where a main effect was seen in Kruskal-Wallis tests, pairwise comparisons between control and each drug treated group were then made using Dunn's multiple comparisons test. In each case, $p<0.05$ was considered statistically significant.

**Results**

**Experiment 1** assessed the effects of harmaline versus a single, $dH_2O$-treated control group. Harmaline reliably induced a significant and persistent tremor that affected all body parts (median tremor score: harmaline: 3 (0.25); control: 0 (0); $p<0.05$; Fig. 1A) and also significantly reduced rearing (median rearing events: harmaline: 0 (1); control: 28 (10); $p<0.05$; Fig. 1B) and grooming events (median grooming events: harmaline: 1(1); control: 2.5 (3.75); $p<0.05$; Fig. 1C). In the open field test, harmaline significantly decreased total distance moved (median distance moved: harmaline: 1097 (373) cm; control: 2734 (297) cm; $p<0.05$; Fig 1D) while mean mobility duration (harmaline: 2.0±0.4 s; control: 4.9±0.7 s; $p<0.05$; Fig. 1E) and median speed (harmaline: 3.7 (1.4) cm $s^{-1}$; control: 9.1 (2.2) cm $s^{-1}$; $p<0.05$; Fig. 1F) were also significantly decreased by treatment. In the rotarod test, median time on the apparatus was significantly decreased by harmaline treatment (harmaline: 33.0 (9.4) s; control: 300.0 (14.7) s; $p<0.05$; Fig. 2A) and, similarly, treatment significantly decreased median gripping time in the grip strength test (harmaline: 15.8 (4.0) s; control: 177.5 (32.1) s; $p<0.05$; Fig 2B). When animal gait was assessed, harmaline significantly increased mean gait width (harmaline: 5.9±0.2 cm; control: 2.2±0.0 cm; $p<0.05$; Fig. 2C) and reduced mean right (harmaline: 6.9 ± 0.2 cm; control: 8.4±0.2 cm; $p<0.05$; Fig 2D) and left (harmaline: 6.4±0.2 cm; control: 8.2±0.3 cm; $p<0.05$; Fig 2E) stride length. These results demonstrate that 30mg $kg^{-1}$ harmaline treatment reliably and reproducibly induces severe tremor associated with significant functional deficits that can be detected and assessed using the tasks employed.

**Experiment 2** assessed the effect of CB receptor agonism upon the harmaline-induced symptoms described in **Experiment 1**. The CB receptor agonist, WIN55, 212-2 (0.1, 0.5 & 1mg $kg^{-1}$) or vehicle was administered 30 minutes before harmaline (30mg $kg^{-1}$) and effects assessed behaviourally as previously described. Here, an overall effect of treatment upon median harmaline-induced tremor ($H(3)=12.1$, $p<0.05$; Fig 3A), median rearing events ($H(3)=13.47$, $p<0.05$; Fig 3B) and median grooming events was seen ($H(3)=18.01$, $p<0.05$;

Fig 3C) although subsequent pairwise comparisons only revealed significant effects of higher WIN55, 212-2 doses upon grooming events (WIN55, 212-2 0.5mg $kg^{-1}$: $p<0.05$; WIN55, 212-2 1mg $kg^{-1}$: $p<0.05$; *vs* harmaline plus WIN55, 212-2 vehicle treated controls; Fig. 3C).

In the open field test, no overall effects of treatment upon mean total distance moved ($F_{3, 41}=2.270$, $p>0.05$; Fig 3D) or median mobility duration ($H(3)=4.509$, $p>0.05$; Fig 3E) were seen although mean movement speed ($F_{3, 37}=4.688$, $p<0.05$; Fig 3F) was affected where *post hoc* tests revealed that WIN55, 212-2 1mg $kg^{-1}$ significantly reduced movement speed ($p<0.05$ vs harmaline plus WIN55, 212-2 vehicle treated controls). In the rotarod test, a main effect of treatment upon median time on the rotarod apparatus ($H(3)=14.21$, $p<0.05$) was seen where WIN55, 212-2 caused a dose dependent exacerbation of harmaline effects on this measure (WIN55, 212-2 0.5mg $kg^{-1}$: $p<0.05$; WIN55, 212-2 1mg $kg^{-1}$: $p<0.01$; each *vs* harmaline plus WIN55, 212-2 vehicle treated controls; Fig. 4A). Furthermore, treatment significantly affected median grip strength ($H(3)=20.28$, $p<0.05$) although *post hoc* comparisons revealed that only WIN55, 212-2 0.5mg $kg^{-1}$ significantly reduced gripping time ($p<0.001$ vs harmaline plus WIN55, 212-2 vehicle treated controls; Fig. 4B). Finally, when animal gait was assessed, significant effects of treatment upon median gait width ($H(3)=13.32$, $p<0.05$; Fig. 4C) and median stride length (right stride: $H(3)=17.35$, $p<0.05$; left stride: $H(3)=9.703$, $p<0.05$; Figs 4D&E) were seen. *Post hoc* comparisons with harmaline plus WIN55, 212-2 vehicle treated controls tests revealed that WIN 55,212-2 0.1mg $kg^{-1}$ decreased the harmaline-induced increase in gait width ($p<0.05$) although WIN 55,212-2 1mg $kg^{-1}$ exacerbated the harmaline-induced decrease in right ($p<0.05$), but not left, stride length.

**Experiment 3** assessed the effects of cannabinoid type 1 receptor antagonism upon harmaline-induced symptoms by examining the effects of the $CB_1$ receptor selective antagonists AM251 (1mg $kg^{-1}$) and rimonabant (10mg $kg^{-1}$) when administered 30 minutes before harmaline (30mg $kg^{-1}$) in our battery of behavioural tasks. A significant effect of drug

treatment (H(2)=17.02, p<0.05) on median tremor score was seen and *post hoc* tests revealed that AM251 (p<0.05) and rimonabant (P<0.05; Fig. 5A) significantly reduced tremor scores when compared to harmaline plus vehicle controls. When rearing events were assessed, a main effect of treatment was detected (H(2)=12.86, p<0.05) and revealed that rimonabant significantly increased rearing events when compared to harmaline plus vehicle (p<0.05, Fig. 5B). A significant effect of treatment upon grooming events was also seen (H(2)=19.88, p<0.05) where both antagonists produced significant increases when compared to harmaline plus vehicle (AM251: p<0.05; rimonabant: p<0.05; Fig. 5C). In the open field test, significant effects of treatment was seen on the median total distance moved (H(2)=17.51, p<0.05), mean mobility duration ($F_{2, 27}$=10.84, p<0.05) and mean movement speed ($F_{2, 27}$=3.792, p<0.05). Here, when comparisons were made vs the harmaline plus vehicle group, *post hoc* tests revealed that both AM251 and rimonabant significantly increased total distance moved (AM251: p<0.05; rimonabant: p<0.05; Fig. 5D) and mobility duration (AM251: p<0.05; rimonabant: p<0.05; Fig. 5E) but only rimonabant significantly increased movement speed (p<0.05; Fig 5F).

In the rotarod test, a main effect of treatment upon mean time on the apparatus was seen ($F_{2, 23}$=47.21, p<0.05) that revealed $CB_1$ receptor antagonist treatment to significantly increase times on the rod when compared to harmaline plus vehicle controls (AM251: p<0.01; rimonabant: p<0.05; Fig. 6A). In the grip strength test, a similar effect was seen where the main effect of treatment ($F_{2, 24}$=24.04, p<0.05) arose from significant effects of $CB_1$ receptor antagonism to increase mean grip time (AM251: p<0.05; rimonabant: p<0.05; Fig. 6B). Finally, in our analysis of gait, a significant effect of treatment was seen upon median stride width (H(2)=14.71, p<0.05; Fig. 6C) but not mean stride length (right: $F_{2, 25}$=1.559, p>0.05& left: $F_{2, 25}$=2.685, p>0.05; Figs 6D&E) where *post hoc* tests revealed that $CB_1$ receptor antagonism reduced stride width (AM251: p<0.05; rimonabant: p<0.05 when compared to harmaline plus vehicle controls.

## Discussion

Essential tremor (ET) is the most common movement disorder (Louis *et al.*, 1998), has unmet clinical need (~50% pharmacoresistance) and is most frequently cerebellar in origin. The endocannabinoid system plays an important role in cerebellar function and $CB_1$ receptor expression is at its most abundant in mammalian cerebellum (Miller *et al.*, 2011). However, while behavioural effects of $CB_1$ receptor agonism in healthy laboratory species are well established (Little *et al.*, 1988), $CB_1$ receptor modulation in ET has never been examined. Such a study is important and timely since the endocannabinoid system may represent an unexploited target for ET pharmacotherapy. Moreover, recreational cannabis use and cannabinoid medicine use is increasing, raising exposure risk in ET patients. Finally, recreational abuse of synthetic cannabinoids (typically $CB_1$ receptor agonists) is also increasing, presenting additional risks within the ET patient population (Fox *et al.*, 2004; Gilman *et al.*, 2014; Tudge *et al.*, 2015). We therefore assessed the effects of CB receptor agonism and $CB_1$ antagonism in a murine ET model using five conventional behavioural assessments.

In our first experiment, and consistent with the literature, harmaline reliably induced tremor (Martin *et al.*, 2005) which manifested as notable performance deficits in all of the behavioral tasks employed. Thus, significant reductions in rearing and grooming events, distance moved by animals in the open field test, mobility duration, movement speed, time on rotarod, grip strength, bilateral gait width and stride length. Harmaline produces tremor, the severity of which is reliably dose-dependent and species-specific (Miwa *et al.*, 2006). Notably, studies seeking to detect the effects of agents that are hypothesised to potentiate ET (e.g. caffeine (Al-Deeb *et al.*, 2002)) most commonly employ a lower dose of harmaline (e.g. 10 mg $kg^{-1}$) while conversely, those exploring the potential therapeutic utility of novel agents to treat ET symptoms will most often employ higher harmaline doses (e.g. 30 mg $kg^{-1}$) (Shourmasti *et al.*, 2014). Here, since a severe tremor state was required, upon which only potent ameliorating or exacerbating pharmacological effects of the cannabinodis studied would be revealed, a harmaline dose of 30 mg $^{kg-1}$ was employed. The primary cause of

harmaline-induced tremor is via alteration of synchronous activation of climbing fibres from the inferior olive projecting to cerebellar PC (Kolasiewicz *et al.*, 2009), most likely via repetitive discharge generation in inferior olivary nucleus neurons through potentiation of $Ca_V3.1$ calcium channels responsible for intrinsic oscillatory activity in this neuronal population (Miwa *et al.*, 2011).

One of the most reported effects of cannabis in a survey of MS patients was tremor relief (Koch *et al.*, 2007). However, other studies have reported that cannabis does not improve MS-associated tremor (Fox *et al.*, 2004; Koppel *et al.*, 2014) and static ataxia can be reliably induced by $CB_1$ receptor agonism in dogs and mice (Dewey *et al.*, 1972). In our second experiment, we examined the consequences of CB receptor activation upon harmaline-induced behavioural deficits in rat. Here, CB receptor agonism largely exacerbated harmaline-induced symptoms as demonstrated by reduced grooming events, movement speed and time spent on the rotarod, consistent with $CB_1$ receptor agonist effects in healthy animals (Little *et al.*, 1988). While these effects occurred only at higher doses of WIN55,212-2 and suggested a possible dose-dependent effect, CB receptor agonism also exerted conflicting and apparently dose independent effects upon features of gait. Here, only the lowest dose of WIN55,212-2 partially reverse harmaline-induced changes in stride width yet the highest dose exacerbated right, but not left stride length. Similarly, only the middle dose of WIN55,212-2 exacerbated the harmaline-induced decrease in grip strength which was unaffected by either the lowest or highest doses.

WIN55,212-2 is an agonist that acts at both $CB_1$ and $CB_2$ receptors. While the presence and functional relevance of central $CB_2$ receptors remains controversial (Xi et al., 2011, Morgan et al., 2009), the potential for some of the effects of WIN55,212-2 reported here to have been mediated, wholly or in part, via $CB_2$ receptor activation cannot be ruled out. Overall, CB receptor agonism typically worsened harmaline-induced symptoms as assessed using the behavioural measures employed. While some conflicting results were found in more nuanced tests of motor function (e.g. gait), they did not appear to be dose

dependent and no indication of potential therapeutic benefit was seen in tests which assessed fundamental features of the model (e.g. tremor).

Our previous *in vitro* studies have suggested that $CB_1$ receptor antagonism may be beneficial in movement disorders by reducing CB1 receptor-mediated inhibition of GABA release (Ma *et al.*, 2008). In the present study, we have shown that $CB_1$ receptor antagonism can ameliorate severe ET symptoms and represents the first behavioural evidence of such clinical potential in an established and relevant animal model. Importantly, the two $CB_1$ receptor antagonists tested both significantly decreased harmaline-induced tremor score, showing beneficial effects on the primary behavioural deficit exhibited in this model. Moreover, while not reaching magnitudes comparable with control animal behaviours, both AM251 and rimonabant increased grooming events when compared to animals only treated with harmaline, while rimonabant alone increased rearing events, largely consistent with previous reports (Zavatti M1, 2011). $CB_1$ receptor antagonism also exerted beneficial effects in the open field test where both antagonists tested ameliorated harmaline-induced behavioural deficits in all measured domains (with the exception of AM251 in movement speed). Similarly, both antagonists exerted beneficial effects upon harmaline-induced adverse effects in the rotaroad and grip strength tasks in addition to ameliorating harmaline effects upon stride width but not stride length. Thus, blockade of endocannabinergic tone exerts intrinsic therapeutic benefit in this rodent model of severe ET. Harmaline treatment evokes rhythmic burst-firing activity in the medial and dorsal accessory inferior olivary nuclei that is propagated via climbing fibres to Purkinje cells, before further transmission to deep cerebellar nuclei, brainstem and spinal cord, consistent with our previous observation (Ma et al., 2008) that $CB_1$ receptor antagonism inhibits Purkinje cell firing via blockade of endocannabinergic inhibition of GABA release although the involvement of other, additional, endocannabinoid-mediated processes cannot yet be eliminated.

While a reversal by cannabinoid type 1 receptor antagonism of harmaline effects upon simple motor functions or their exacerbation by $CB_1$ receptor agonists most likely arise predominantly from central $CB_1$ receptor-mediated effects, some $CB_1$ receptor antagonists

exert off target effects. Therefore, and particularly with regard to results where a clear dose-related response was not evident, further investigation is warranted to determine potential interplay between such signaling systems. *In vitro*, rimonabant and AM251 can allosterically potentiate $GABA_A$ receptors at nanomolar concentrations although their site of action is distinct from other allosteric modulators of this receptor (Baur *et al.*, 2012; Giovanni Battistella, 2014 ). Moreover, GlyRs are involved in a number of movement disorders (Yang *et al.*, 2008) and exhibit a distinct pharmacological profiles for several cannabinoid compounds and cannabinoid receptor ligands and so establish GlyRs as novel targets for endogenous and exogenous cannabinoids (Yang *et al.*, 2008).

Similar to the cerebellum and ET, $CB_1$ receptor expression is also abundant in the cerebral ganglia (Pacher *et al.*, 2009) and has been studied in a primate model of dyskinesia. Here, while rimonabant reduced dyskinetic symptoms (van der Stelt *et al.*, 2005), another $CB_1$ receptor antagonist, 1-[7-(2-chlorophenyl)-8-(4-chlorophenyl)-2-methylpyrazolo[1,5-a]-[1,3,5] triazin-4-yl]-3-ethylaminoazetidine-3-carboxylic acid amide benzenesulfonate failed to affect dyskinetic symptoms (Cao *et al.*, 2007). Moreover, the CB receptor partial agonist, nabilone, also alleviated symptoms in the same model (Fox *et al.*, 2002) and in a small clinical pilot (Sieradzan *et al.*, 2001) but these results were not replicated in a randomized-controlled clinical trial (Carroll *et al.*, 2004). Thus, as found in the present study with respect to $CB_1$ receptor modulation of ET symptoms, other dyskinesias appear either improved or unaffected by $CB_1$ receptor antagonism but paradoxically alleviated and exacerbated by CB receptor agonism. This contradiction, exemplified by our own results and those describing therapeutic benefits of CB1 receptor agonism in animal models of chronic tremor (Koch *et al.*, 2007; Baker et al., 2000) may suggest that overall effects are determined by the aetiology of the disorder modelled. Thus, in a chronic encephalomyelitis modelling multiple sclerosis (Baker et al., 2000) where widespread demyelination and axon loss occurs, CB receptor agonism can be of use while in acute

tremor arising from cerebellar hyperexcitability (e.g. harmaline treatment) to model idiopathic ET, $CB_1$ receptor antagonism is beneficial.

In conclusion, our results demonstrate that acute $CB_1$ receptor antagonism improves severe ET symptoms and so demonstrates their therapeutic potential for ET. Rimonabant was previously licensed for weight loss although was withdrawn in 2008 following reports of psychiatric side effects in a trial population where higher doses were employed (Moreira *et al.*, 2009). However, adverse reactions of this nature do not necessarily preclude the use of a treatment (e.g. suicidal ideation associated with SSRIs) (Ghaziuddin *et al.*, 2014) and so should not hinder drug development if warranted by unmet clinical. Moreover, rimonabant has since been shown to act as an inverse agonist at $CB_1$ receptors (Landsman *et al.*, 1997) and so making investigation neutral $CB_1$ receptor antagonists (e.g. $\Delta^9$-tetrahydrocannabidavarin (Tudge *et al.*, 2015)) in ET compelling since it is already known to modulate PC firing *in vitro* (Ma *et al.*, 2008).

Our study reinforces the pivotal role of the endocannabinoid system in motor function and highlights its therapeutic potential in the treatment of ET symptoms. Our novel findings justify further study of the basic neuronal circuits that subserve $CB_1$ receptor antagonist therapies for ET alongside further *in vivo* studies to elucidate mechanisms of $CB_1$ receptor antagonist effects on harmaline symptoms (e.g. central microdialysis). Moreover, while harmaline-induced tremor is a valuable first line model used to inform prioritisation of candidate ET treatments for subsequent investigation it is necessarily limited as a result of its acute nature. Harmaline-induced tremor is predictive of clinical efficacy in ET in ~50% of cases (Handforth, 2012) and so the findings presented here strongly support further preclinical study of repeated $CB_1$ receptor antagonist treatment in animal models disease (c.f. models of acute symptoms as used here) and subsequent clinical development.

**Contributions:**

Hassan Abbassian: Execution of research project, statistical analysis, manuscript preparation

Benjamin J. Whalley: Conception, organization, review and critique of research, statistical analysis, manuscript preparation.

Vahid Sheibani: Organization of research project.

Mohammad Shabani: Conception and organization of research project, statistical analysis, manuscript preparation

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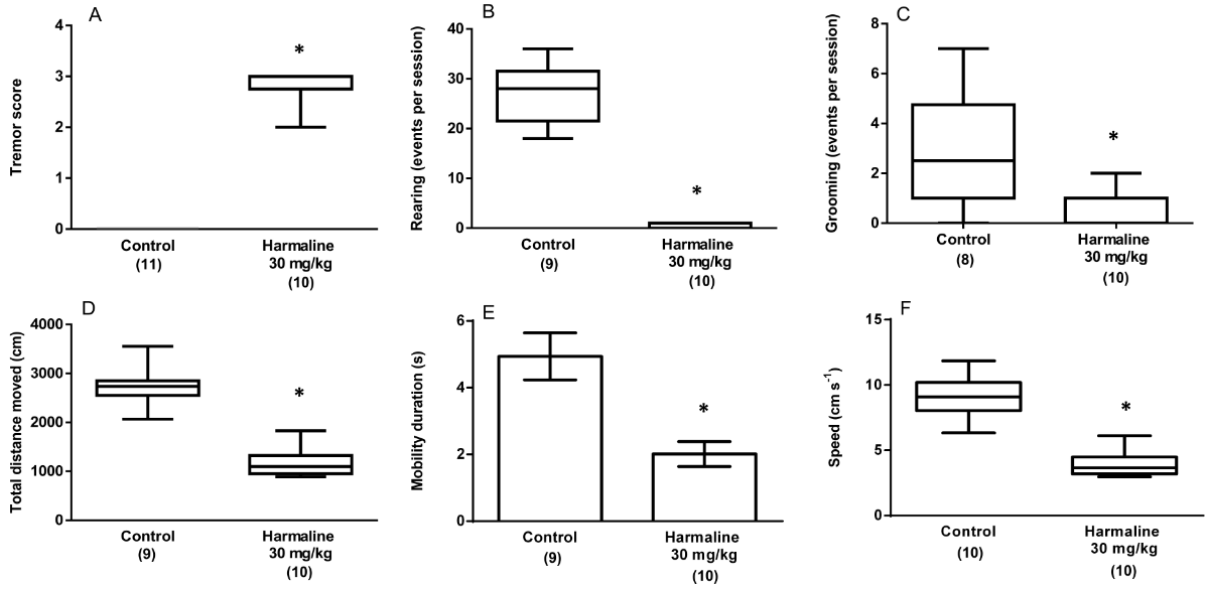


**Figure 1.** Experiment 1: The effect of harmaline (30 mg $kg^{-1}$; i.p.) upon (**A**) tremor score, (**B**) rearing events per session and (**C**) grooming events per session. Results from the same treatment in the open field test are shown as (**D**) total distance moved (cm), (**E**) mobility duration (s) and (**F**) movement speed (cm $s^{-1}$). Data describing mobility duration exhibited a normal distribution and are represented as mean±SEM. Data describing tremor score, rearing events, grooming events, total distance moved and movement speed were not normally distributed and are represented as medians with interquartile ranges as a box and maxima/minima as whiskers. *= $p<0.05$ where all comparisons were made with the vehicle ($dH_2O$; i.p) treated control group. Numbers in parentheses indicate group sizes. No data points were excluded as outliers in the presented analyses.

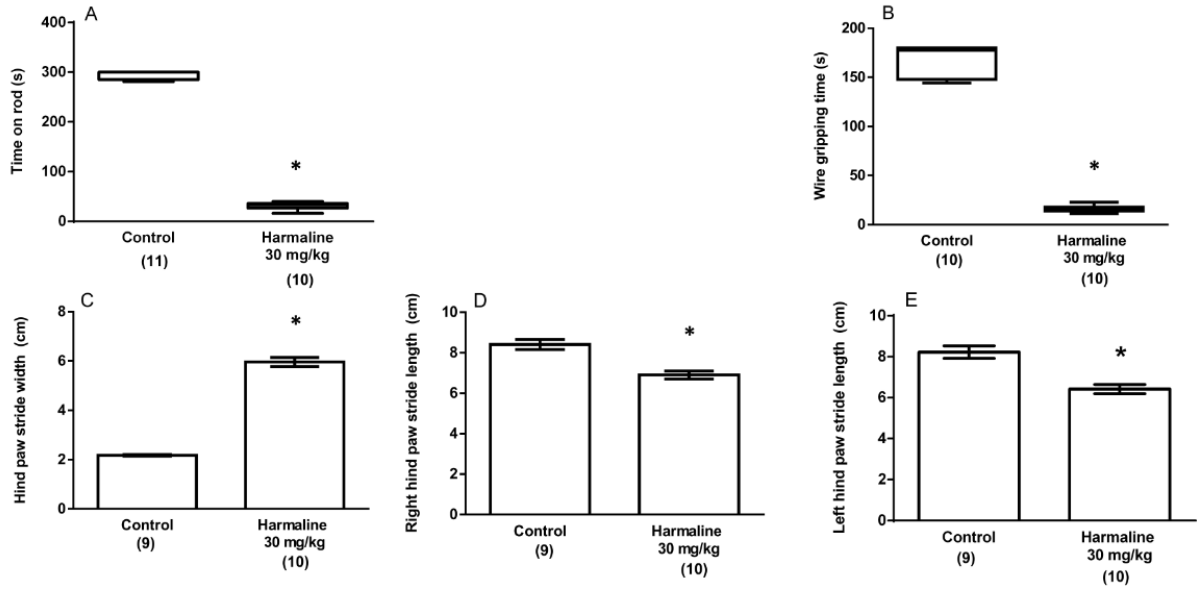


**Figure 2.** Experiment 1: The effect of harmaline (30 mg $kg^{-1}$; i.p.) upon (**A**) time spent on rotarod apparatus and (**B**) gripping time in the wire grip test. Results from the same treatment in the gait analysis test are shown as (**C**) hind paw stride width (cm), (**D**) right hind paw stride length (cm) and (**F**) left hind paw stride length (cm). Data describing measures from the gait analysis exhibited a normal distribution and are represented as mean±SEM. Data describing time on the rotarod apparatus and gripping time in the wire grip test were not normally distributed and are represented as medians with interquartile ranges as a box and maxima/minima as whiskers. *= $p<0.05$ where all comparisons were made with the vehicle ($dH_2O$; i.p) treated control group. Numbers in parentheses indicate group sizes. No data points were excluded as outliers in the presented analyses.

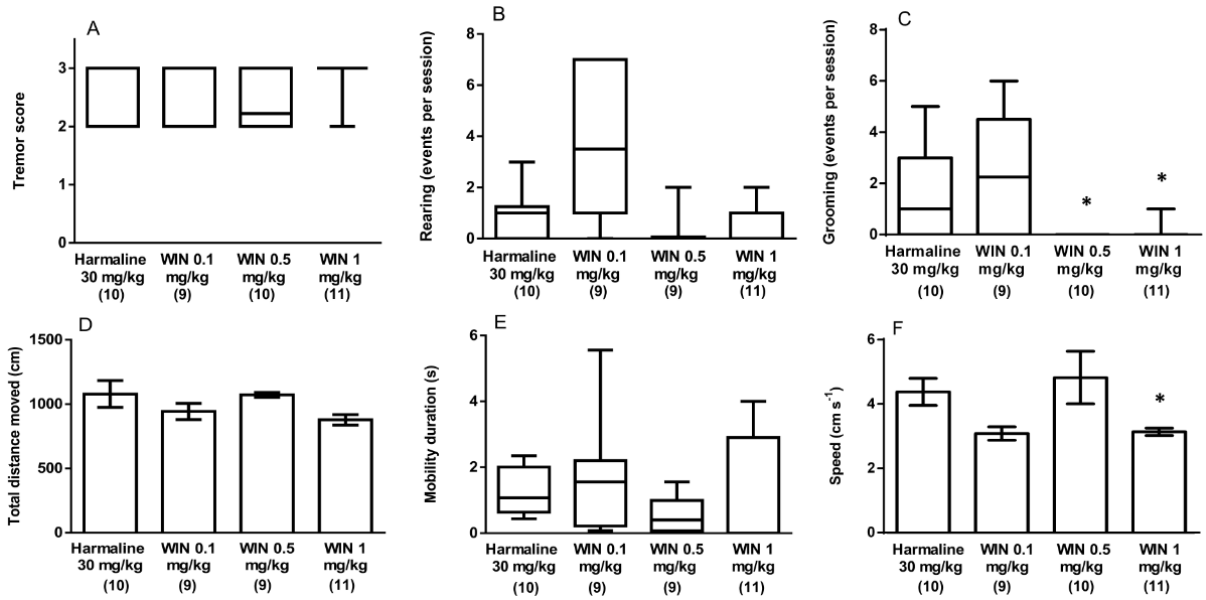


**Figure 3.** Experiment 2: The effect of CB receptor agonist (WIN55,212-2 0.1, 0.5 & 1 mg $kg^{-1;}$ i.p.) treatment upon harmaline (30 mg $kg^{-1}$; i.p.) induced symptoms. (**A**) tremor score, (**B**) rearing events per session and (**C**) grooming events per session. Results from the same treatment in the open field test are shown as (**D**) total distance moved (cm), (**E**) mobility duration (s) and (**F**) movement speed (cm $s^{-1}$). Data describing total distance moved and movement speed exhibited a normal distribution and are represented as mean±SEM. Data describing tremor score, rearing events, grooming events and mobility duration were not normally distributed and are represented as medians with interquartile ranges as a box and maxima/minima as whiskers. *= $p<0.05$ where all comparisons were made with the harmaline only group. Numbers in parentheses indicate group sizes. No data points were excluded as outliers in the presented analyses.

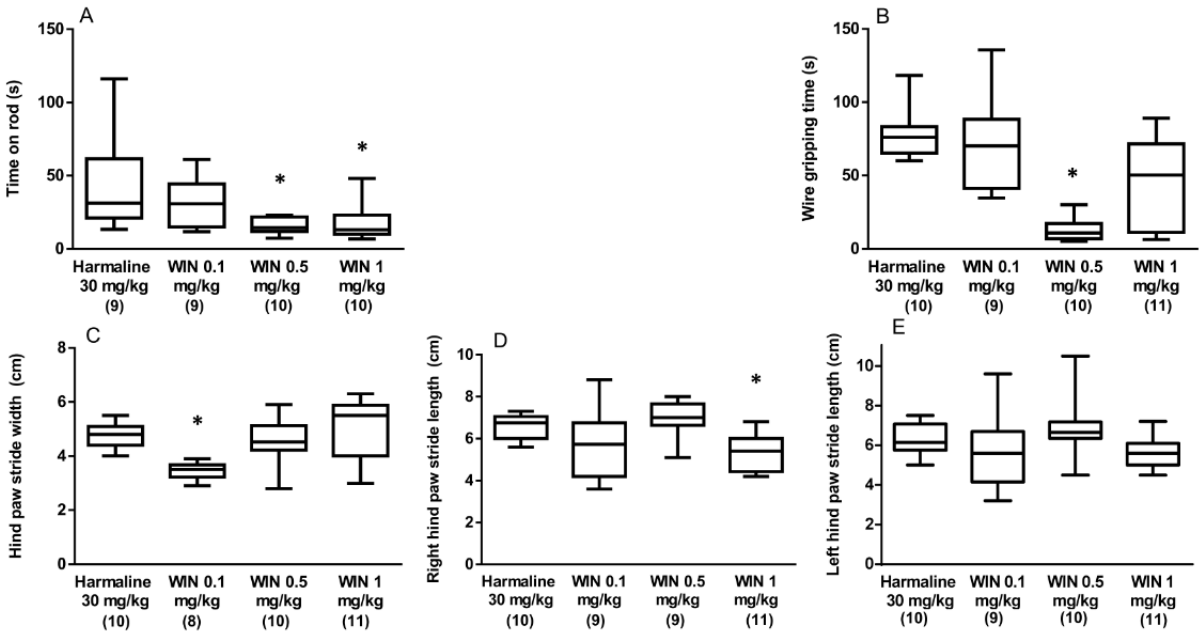


**Figure 4.** Experiment 2: The effect of CB receptor agonist (WIN55-212,2 0.1, 0.5 & 1 mg $kg^{-1}$; i.p.) treatment upon harmaline (30 mg $kg^{-1}$; i.p.) induced symptoms. (**A**) time spent on rotarod apparatus and (**B**) gripping time in the wire grip test. Results from the same treatment in the gait analysis test are shown as (**C**) hind paw stride width (cm), (**D**) right hind paw stride length (cm) and (**F**) left hind paw stride length (cm). Data for all measures in this experiment were not normally distributed and are represented as medians with interquartile ranges as a box and maxima/minima as whiskers. *= $p<0.05$ where all comparisons were made with harmaline only group. Numbers in parentheses indicate group sizes. 3/199 data points were detected as outliers and excluded from the presented analyses.

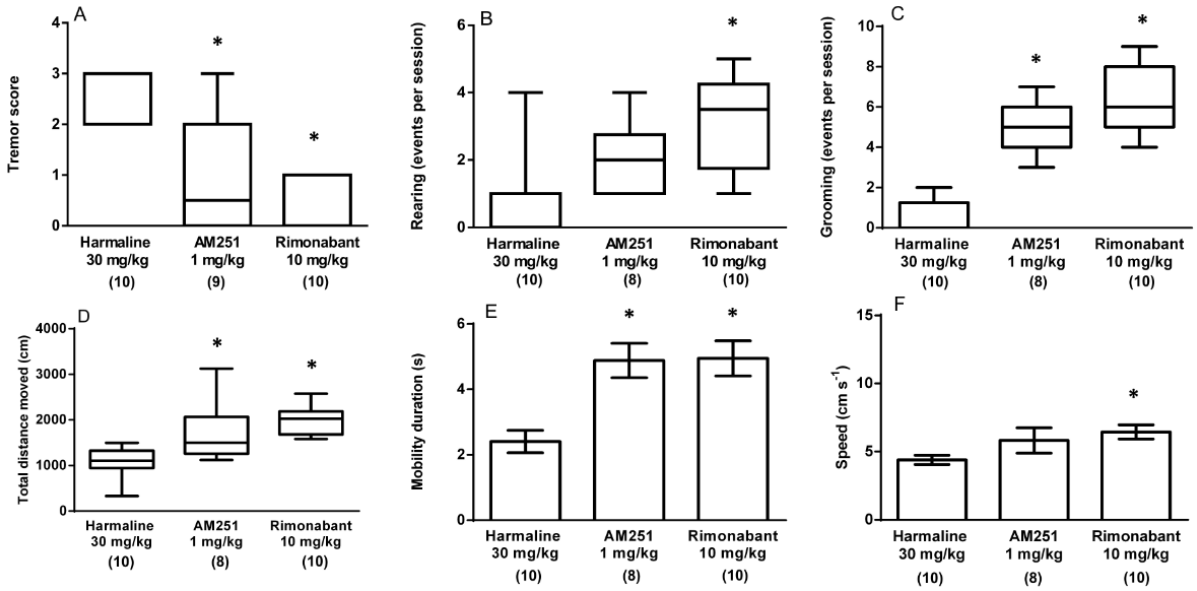


**Figure 5.** Experiment 3: The effect of CB1 antagonist (AM251 1 mg $kg^{-1}$ and rimonabant 10 mg $kg^{-1}$; both i.p.) treatment upon harmaline (30 mg/kg; i.p.) induced symptoms. (**A**) tremor score, (**B**) rearing events per session and (**C**) grooming events per session. Results from the same treatment in the open field test are shown as (**D**) total distance moved (cm), (**E**) mobility duration (s) and (**F**) movement speed (cm $s^{-1}$). Data describing mobility duration and movement speed exhibited a normal distribution and are represented as mean±SEM. Data describing tremor score, rearing events, grooming events and total distance moved were not normally distributed and are represented as medians with interquartile ranges as a box and maxima/minima as whiskers. *= $p<0.05$ where all comparisons were made with the harmaline only treated group. Numbers in parentheses indicate group sizes. 3/172 data points were detected as outliers and excluded from the presented analyses.

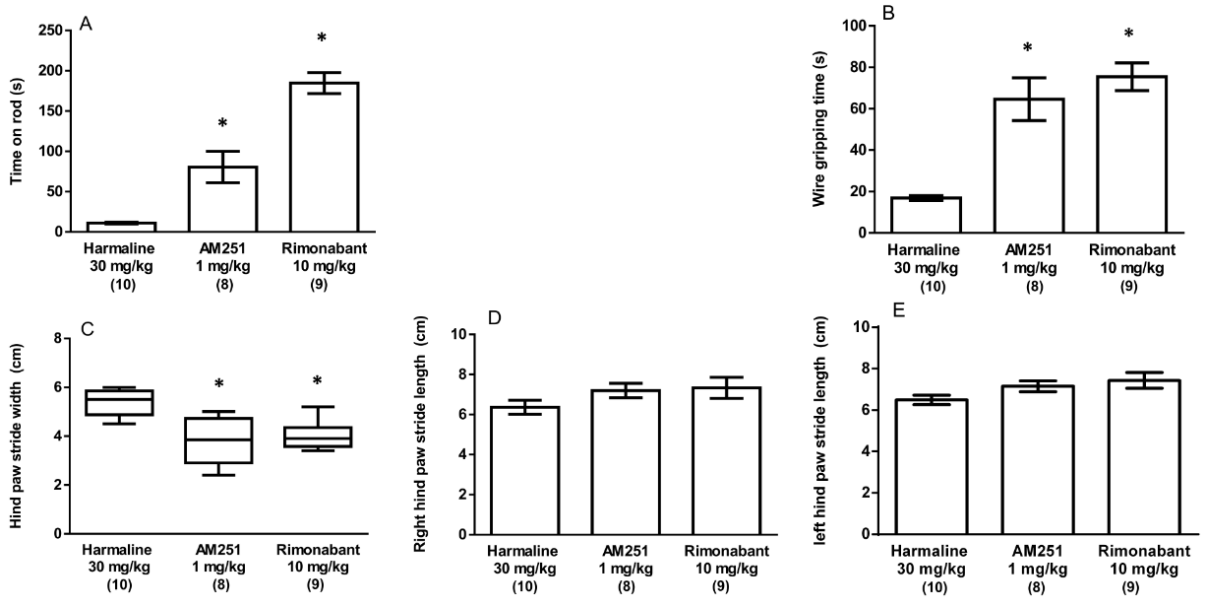


**Figure 6.** Experiment 3: The effect of CB1 antagonist (AM251 1 mg $kg^{-1}$ and rimonabant 10 mg $kg^{-1}$; both i.p.) treatment upon harmaline (30 mg $kg^{-1}$; i.p.) induced symptoms. (**A**) time spent on rotarod apparatus and (**B**) gripping time in the wire grip test. Results from the same treatment in the gait analysis test are shown as (**C**) hind paw stride width (cm), (**D**) right hind paw stride length (cm) and (**F**) left hind paw stride length (cm). Data for time on rotarod apparatus, gripping time in the wire grip test, and right and left hind paw stride lengths were normally distributed and are represented as mean±SEM. Hind paw stride width data were not normally distributed and are represented as medians with interquartile ranges as a box and maxima/minima as whiskers. *= $p<0.05$ where all comparisons were made with harmaline only group. Numbers in parentheses indicate group sizes. No data points were excluded as outliers in the presented analyses.