Assessing physiological dependence and withdrawal potential of mitragynine using schedule-controlled behaviour in rats

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Conflict of interest

None

Abstract

Rationale: Kratom is proposed to exhibit therapeutic potential as an opium substitute but little is known about its dependence-producing profile, particularly of its main psychoactive compound, mitragynine (MG).

Objectives: This study examined the dependence-producing effects of MG using operant-scheduled behaviour in rats and investigated the potential therapeutic effect of MG by comparing effects to buprenorphine in morphine-dependent rats using the same schedule-controlled behavioural task.

Methods: The effects of acutely-administered MG and morphine were determined in rats trained to respond under fixed-ratio (FR) 10 schedule of food reinforcement. Next, the rats were administered MG and morphine twice daily for 14 consecutive days to determine if physiological dependence would develop by examining cessation of drug treatment and following antagonist-precipitated withdrawal. The study then examined the effects of MG substitution to suppress naloxone-precipitated morphine withdrawal effects on scheduled responding.

Results: Acute doses of MG did not produce dose-related decreases on FR schedules of responding compared to morphine. Unlike morphine, MG-treated rats showed no suppression of response rates following cessation of MG treatment. However, withdrawal effects were evident for MG after precipitation by either naloxone or SR141716A (rimonabant), similar to morphine-treated rats. MG in higher doses (10 and 30 mg/kg) attenuated the naloxone-precipitated morphine withdrawal effects while smaller doses of buprenorphine (0.3 and 1.0 mg/kg) were necessary to alleviate these effects.

Conclusion: The findings suggest that MG does not induce physiological dependence but can alleviate the physical symptoms associated with morphine withdrawal which represent the desired characteristics of novel pharmacotherapeutic interventions for managing opioid use disorder (OUD).

Keywords: Mitragynine, Kratom, Opioid, Schedule-Controlled Behavior, Rat, Morphine, Naloxone, Withdrawal, Dependence

Introduction

Opioid abuse has become a major global issue (Compton et al. 2006) that can lead to physiological dependence, a condition in which cessation of drug use results in the manifestation of overt withdrawal symptoms. Repeated use of opioids is usually associated with dependence, a condition which also results in overt withdrawal symptoms upon cessation of drug use. The desire to avoid these unpleasant symptoms of withdrawal is one of the main reasons for opioid seeking, and potentially relapse (Volkow et al. 2016). The commonly prescribed medications for the treatment of opioid dependence such as methadone and buprenorphine have the effect of reducing opioid withdrawal symptoms. However, they are associated with certain compliance issues and, more significantly, with side-effects, such as the further risk of abuse potential and relapse (Gonzalez et al. 2004; Volkow et al. 2016). This situation highlights the urgent need to develop safer alternative medications to treat opioid dependence.

Mitragynine (MG), the psychoactive phytochemical derived from *Mitragyna speciosa* Korth or kratom, is receiving global attention, particularly for its use in the self-treatment and managing of opioid withdrawal syndrome (Grundmann 2017). Known to the locals as 'ketum', 'biak-biak' or 'krathom', this tropical medicinal plant is indigenous to Southeast Asian regions whereby the leaves are consumed by either brewing the leaves into tea or by chewing them fresh (Hassan et al. 2013). It has been used traditionally to treat minor ailments, to increase physical endurance for manual laborers and to enhance mood and well-being (Assanangkornchai et al. 2007; Saingam et al. 2013; Vicknasingam et al. 2010). However, in addition to its medicinal and social benefits, kratom was also used in the management of opioid withdrawal symptoms of chronic opioid users who were temporarily without access to opioids and were trying to give up either opioids, alcohol or other addictive drugs (Vicknasingam et al. 2010; Singh et al. 2017). Reports on the use of kratom as a substitute for opioid withdrawal syndrome as well as its use for weaning addicts off morphine have become more widespread in recent years and extend beyond the natural geographical boundaries of the plant (Boyer et al. 2008; Grundmann 2017; Swogger and Walsh 2018). Given the relative ease of purchasing kratom via online platforms, kratom products are now globally sold as herbal supplements, in the form of powder, pills, capsules or energy drinks.

MG is the main active alkaloid which accounts for 66% of the total alkaloid content extracted from the leaves of *Mitragyna speciosa* Korth (Shellard 1974; Ponglux et al. 1994; Hassan et al. 2013). Studies have demonstrated that most of pharmacological effects of MG are exerted via the opioid receptor system, primarily by action on the μ-opioid receptor subtype (Matsumoto et al. 1996, Yusoff et al. 2017). The fact that MG substitutes for the morphine discriminative stimulus (Harun et al. 2015) and can attenuate morphine withdrawal-induced anxiety in zebrafish (Khor et al. 2011) suggest that MG could be an effective substitute in the management of morphine dependence. While some users report negative effects, particularly nausea and vomiting resulting from the ingestion of higher kratom doses, the majority of users report on beneficial effects of kratom as a form of self-medication for pain and relief of opioid withdrawal symptoms (Swogger et al. 2015; Singh et al. 2017). Although the therapeutic potential of kratom appears promising, several major therapeutic issues remain, including its dependence liability which have not been systemically evaluated. Previous studies of rodents have demonstrated little or no abuse liability of MG (Harun et al. 2015; Hemby et al. 2018; Yue et al. 2018) which suggests a potential therapeutic use of MG for treating opioid dependence. Although there are comprehensive data available on the pharmacological properties of MG, particularly on its opioid-like analgesic effects (Matsumoto et al. 1996; Thongpradichote et al. 1998; Takayama et al. 2002), the mechanisms underlying its abuse and dependence liabilities remain unclear.

It is well-known that the repetitive use of psychoactive substances can induce physiological dependence, as evidenced by behavioural disruption upon abstinence. In laboratory animals, physiological dependence upon psychoactive compounds is characterized by the emergence of withdrawal effects such as changes on conditioned behaviour (Balster 1985). For this reason, schedule-controlled operant behaviour is used to detect withdrawal signs in laboratory rodents. This procedure involves assessment of rates of food-reinforced schedule-controlled behavior in which suppression of the response rates represents the key index of drug-induced dependence (Ford and Balster 1976; Adams and Holtzman 1990, Schulteis et al. 1997).

The present study aimed to investigate the physiological aspects of MG withdrawal as measured by the disruption of behavioural performance in rats. The presence of physiological dependence was tested during spontaneous withdrawal or following precipitation of withdrawal with naloxone, an opioid receptor antagonist. Since Ismail et al. (2017) reported that behavioural alteration induced by MG was similar to the effects of morphine and Δ-9-tetrahydrocannabinol (THC), the present study also investigated whether the CB-1 cannabinoid receptor antagonist, SR141716A (rimonabant) could also precipitate MG withdrawal. The subsequent study was conducted to determine whether MG could ameliorate the symptoms of morphine withdrawal. Hence, this study may provide pre-clinical evidence towards the assessment of whether MG has the therapeutic potential to manage opioid dependence.

Materials and methods

Animals

Male Sprague Dawley rats initially weighing between 200-250g were housed individually in a holding room illuminated under a 12-h light/dark cycle. Access to food was limited to maintain the body weight at 85% of those under free-feeding conditions and to motivate lever-press training. Water was available *ad libitum* throughout the experiment. The experiments were conducted within the bounds of local ethical regulations and were carried out in accordance to the guidelines for the use of experimental animals and approved by the Animal Ethics Committee, Universiti Sains Malaysia (AECUSM).

Apparatus

The experiment utilized six identical two-lever standard operant conditioning chambers (Med-Associates, Vermont, US). Each chamber consisted of a Plexiglas TM enclosure equipped with one house light to provide general illumination, a fan to provide ventilation and to mask extraneous sounds. The operant panel of the chamber consisted of two levers (4.5 cm wide, extending 2.5 cm from the aluminium wall and located 7.0 cm above the floor) which were equidistant from a food receptacle where a dispenser delivered food pellets. Completion of a specified number of responses on the active lever resulted in activation of the pellet dispenser, which delivered a single 45-mg food pellet (BioServ, New Jersey, US) into the food receptacle within the chamber. The house light signalled food availability, and it was turned off during food presentation and the time-out periods. The operant conditioning chambers were controlled by a computer using the MED-PC software package (MED-Associates, Vermont, US).

Drugs

MG was extracted, isolated and verified from fresh leaves of *Mitragyna speciosa* at the Malaysian Institute of Pharmaceuticals and Neutraceuticals, Universiti Sains Malaysia as described previously (Utar et al. 2011). The purity of MG obtained was approximately 98% as confirmed by HPLC and nuclear magnetic resonance (^{1}H-NMR) analyses, which were performed in the analytical laboratory at Centre for Drug Research, Universiti Sains Malaysia. The compound was kept at 4˚C until the time of the experiment. Morphine hydrochloride was purchased from Pharmaniaga Logistics SDN BHD (Malaysia) while naloxone hydrochloride and rimonabant hydrochloride were obtained from Sigma Chemical Co. (USA). All drugs were dissolved in physiological saline (0.9% NaCl) containing 20% (v/v)

Tween-80 and MG was diluted to the desired concentrations prior to the experiment. All drugs and vehicle injections were administered in a volume of 1.0 ml/kg per body weight by the intraperitoneal route. Conversion of animal (i.e. rats) dose to human equivalent dose (HED) was calculated by dividing the animal dose by 6.2 (Nair and Jacob 2016).

Experimental procedure

Operant training

The training procedure for schedule-controlled behavior in rats was adapted from previous publications (Gunter et al. 2015; Sieman et al. 2016). Rats (n= 30) were initially trained to press the right lever for food under a fixed-ratio (FR) schedule. During the shaping phase, one lever was randomly assigned as active and the other lever was inactive. Presses on the active lever resulted in the delivery of a 45-mg food pellet while presses on the inactive lever were recorded but had no programmed consequence. Initially, a single response produced a food pellet (FR-1). Then, as performance improved, the response requirement was progressively increased across days to a final fixed-ratio of 10 (FR-10). The rats were trained daily for 15-min experimental sessions from Mondays to Fridays to facilitate a stable level of responding on FR-10 for all rats. Baseline stability was determined according to a stability criterion in which response rates were not to vary by more than ±10% of the average response rate over the last five consecutive sessions. Once each rat satisfied the stability performance criterion, the next phase of testing began.

Experiment 1 (a): Acute effects of MG on schedule-controlled responding

Once baseline stability was met for all rats, they were randomly allocated into three experimental groups (MG, morphine and vehicle control) which consisted of 8 to 10 rats per group. Each rat was tested with a randomized sequence of acute doses of MG (0, 5.6, 10, 18, 30 and 50 mg/kg, i.p.) and morphine (0, 3, 5.6, 10 and 18 mg/kg, i.p.). The dose range for testing acute doses of morphine was based on previous reports (Ford and Balster 1976; Li et al. 2010; An et al. 2012), while the doses of MG were selected from previous studies assessing various behavioural effects of MG (Shamima et al. 2012; Sabetghadam et al. 2013; Harun et al. 2015). On test days, the rats were injected with vehicle or a randomized sequence of MG and morphine doses 30-mins pre-session and then placed in the operant conditioning chambers and tested under the FR-10 schedule conditions. The timing of sessions for both MG and morphine were selected based on previous studies (Harun et al. 2015; Yusoff et al. 2016; Yusoff et al. 2017). For the control group, the rats were administered a vehicle injection, 30 mins before the operant conditioning sessions. Each rat was tested with all the graded doses of the drug or vehicle and was tested twice per week with at least two training sessions preceding each test session.

Experiment 1 (b): Assessment of spontaneous MG withdrawal

After a washout period of at least 2 weeks following the last acute drug administration (during which training sessions continued), the same group of animals was re-assigned into MG-dependent, morphine-dependent and vehicle control groups (n=8-10). A new baseline was determined for all groups prior to the administration of the drug and the vehicle. The rats were administered drugs twice daily to induce physiological dependence (Tsou et al. 1995) while escalating doses were administered so that opioid tolerance did not develop before the doses were increased (Thorn et al. 2016).

Rats in the MG-dependent group were administered with escalating doses of MG twice daily for 14 days (15, 20, 25, 35 and 45 mg/kg, i.p.) at 0900 and 1800 hrs. Experimental sessions were initiated at 1500 hrs, 6 hrs after the morning injection (Ford and Balster 1976). An initial dose of 15 mg/kg was administered twice on the first 2 days, followed by 20 mg/kg for the next 2 days. Similarly, this was followed by doses of 25 mg/kg for the next 3 days, then by 35 mg/kg for the next 3 days and finally followed by 45 mg/kg for the next 4 days. The timing of the twice daily doses of MG was similar to the morphine dosing regimen (Anraku et al. 2001; Cooper et al. 2008; Salmanzadeh et al. 2017).

Similarly, in the morphine-dependent group, the rats were treated with escalating doses of morphine twice daily for 14 days (5, 7.5, 10, 12.5 and 15 mg/kg, i.p.) at 0900 and 1800 hrs, while the experimental sessions were initiated at 1500 hrs, 6 hrs after the morning injection (Ford and Balster 1976). The initial dose of 5 mg/kg was administered twice on the first 2 days, followed by 7.5 mg/kg for the next 2 days. Then, by 10 mg/kg for the next 3 days, followed by 12.5 mg/kg for the next 3 days and finally, by 15 mg/kg for the next 4 days. This morphine dosing regimen was based on previous reports (Anraku et al. 2001; Cooper et al. 2008; Salmanzadeh et al. 2017) used to establish morphine dependence in rats but with a slight modification to comply with the approximate 1:3 ratio of morphine to MG doses which was extrapolated from MG discrimination data (Harun et al. 2015).

After the 14-day protocol, a single dose of MG or morphine was injected on the morning of the fifteenth day. For control groups, the rats were injected twice daily with the vehicle for the first 14 days and then with a single vehicle injection on the fifteenth day. Dependency on the drug was assessed at every 12-hr interval after the last MG, morphine and vehicle injections (day 15) for 2 to 3 days.

Experiment 1 (c): Assessment of naloxone-precipitated MG withdrawal

After a washout period of 2 weeks (during which training sessions continued), the effects of physiological dependence were again determined using the same group of animals. A new baseline was determined for all groups prior to the administration of the drugs and the vehicle. The rats were treated similarly using the 14-day protocol of inducing MG and morphine dependence as described above, except that 1 mg/kg naloxone (i.p.) was administered 2 hrs after the last injection of MG, morphine and vehicle on day 15. Food-maintained operant responding was assessed for 30 mins following naloxone administration and at every 12-hr interval for up to 2 to 3 days.

Experiment 1 (d): Assessment of rimonabant-precipitated MG withdrawal

A subsequent experiment was conducted to see if rimonabant would precipitate withdrawal after repeated exposure of MG or morphine to rats (14-day treatment). Following a washout period of 2 weeks (during which training sessions continued), a new baseline was re-established for all groups prior to the administration of the drug and vehicle. The design of the experiment was similar to the 14-day protocol used to induce MG and morphine dependence as described above. Following twice daily injections of MG and morphine for 14 days, the rats were given a single injection of MG or morphine on the morning of day 15, 2 hrs before the challenge with rimonabant (1 mg/kg, i.p.). A dose of 1 mg/kg of rimonabant (i.p.) was administered 2 hrs after the last injection of MG, the morphine and the vehicle (day 15). Food-maintained operant responding was assessed at 30 mins following rimonabant administration and tested at 12-hr intervals for up to 2 to 3 days.

Experiment 2: Efficacy of MG and buprenorphine to suppress naloxone-precipitated morphine withdrawal

Drug naïve rats (n= 24) were trained to lever press using a similar procedure as described previously (Gunter et al. 2015; Sieman et al. 2016). Once baseline stability was achieved for all rats, they were randomly divided into two groups. One group of rats (n=12) was treated similarly using the 14-day protocol (5, 7.5, 10, 12.5 and 15 mg/kg, i.p.) of morphine dependence induction while the other group (n=12) was treated with the vehicle.

The procedure of repeated testing was adopted in this study. After 14 days of chronic treatment with morphine, a single dose of morphine was injected on the morning of the fifteenth day, while the rats in the vehicle group were injected with vehicle. Naloxone at 1 mg/kg (i.p.) was administered 2 hrs after the last injection of morphine or vehicle (day 15). The rats in both groups were tested with a randomized series of injections; vehicle, 1, 10 and 30 mg/kg dose of MG (i.p.) and 0.1, 0.3 and 1.0 mg/kg of buprenorphine (i.p.), each administered 30 mins after naloxone injection (Stoller et al. 2004; Harun et al. 2015; Yusoff et al. 2016).

The withdrawal-associated behavioural changes were evaluated repeatedly in the same rat while maintaining the magnitude of dependence across substitution test sessions. Therefore, on each subsequent test, the dose of morphine was escalated by 2.5 mg/kg until the final test, in which animals received the dose of 27.5 mg/kg. The cumulative morphine doses (17.5, 20.0, 22.5, 25.0 and 27.5 mg/kg) were administered twice daily (0900 and 1800 hrs) for every consecutive 4 days followed by a single administration on the next morning until all rats had been tested with the randomised sequences of MG and buprenorphine substitution doses. The effects of MG and buprenorphine were tested for their ability to reduce the intensity of naloxone-precipitated morphine withdrawal.

Data analysis

The number of presses on the active lever was recorded during each session and served as the main dependent measure. The rate of operant responding was expressed as a percentage of the control response rate (% control) and mean responses. The data were analysed by repeated measures analysis of variance (ANOVA) with Bonferroni post hoc tests comparing each dose to the vehicle control. These analyses were conducted using SPSS version 24. In all instances of data analyses, differences were considered significant if $p<0.05$.

Results

Acute effects of MG on schedule-controlled responding

Figure 1 illustrates the effects of an acute challenge with MG and morphine on food-maintained lever-pressing response rates. Acute administration of MG (5.6 – 50 mg/kg, i.p.) to naïve rats did not significantly alter total response rates compared to vehicle administration (Figure 1a). Statistical analyses did not reveal an overall effect of MG dose ($F_{5,48}=0.481, p=0.789$), while the response rate at 30 mins following the highest MG dose (50 mg/kg) was reduced to approximately 75% of control levels but this effect was not significant *($F_{1,16}=2.783$, $p=0.115$).*

As shown in Figure 1b, the administration of 3.0 mg/kg morphine did not markedly affect the rate of responding as there was no significant difference when compared to the baseline response rate ($F_{1,6}=0.071$, $p=0.798$). Meanwhile,

the rats administered with 5.6 mg/kg morphine exhibited a significant decrease of around 50% of the baseline levels of responding ($F_{1,6}$=11.339, p=0.015), while both 10 mg/kg and 18 mg/kg of morphine produced substantial decreases on rates of responding close to 0% of control levels ($F_{1,6}$=69.723, p=0.0005) and ($F_{1,6}$=72.805, p=0.0005), respectively. Taken together, rats in the morphine group demonstrated dose-dependent decreases on lever press response rates ($F_{4,33}$=4.154, p=0.0005).

Assessment of spontaneous MG withdrawal

Baseline levels of performance preceding chronic drug exposure, expressed as mean response rates, are shown in the leftmost disconnected point of Figure 2. These values were obtained from the vehicle administration on the day immediately preceding chronic drugs or vehicle administration. When treated with the vehicle, the operant response rate was relatively stable over this phase of the experiment as there were no significant differences in rates of responding as a function of days ($F_{14,70}=1.351, p=0.201$). Post hoc analyses indicated no significant differences during the test days compared to baseline levels.

Rats in the MG-treated group showed no significant difference in their response rates as a function of days ($F_{21,126}=1.407, p=0.127$). Following the cessation of MG treatment, a small reduction (20%) in response rate was evident during the first 12-hr but this effect was not significantly different from the baseline ($F_{1,5}=0.802, p=0.412$). Although there was a visible decrease in responding in the first 12-hr after MG discontinuation, when compared to the vehicle-treated group at the same time point, this between group comparison was not significantly different ($F_{1,5}=3.880, p = 0.106$).

In contrast, significant reductions were evident in the group chronically exposed to morphine which resulted in significant reductions in response rates as a function of days ($F_{20,140}=8.04, p=0.0005$). The response rate during the first 12-hr of post-morphine treatment was reduced to approximately 40% of the baseline rate of responding ($F_{1,7}=52.538, p=0.0005$). Furthermore, post hoc tests revealed that the response rates were significantly decreased at 24- and 36-hr intervals following cessation of morphine treatment in which response rates were reduced to approximately 55% and 60% of the baseline rate of vehicle responses ($F_{1,7}=27.79, p=0.001; F_{1,7}=77.73, p=0.0005$), respectively. However, the response rates for the remaining 12-hr interval were not significantly different from the vehicle-treated baseline level.

Assessment of naloxone-precipitated MG withdrawal

The effects of 14 days of chronic drugs or vehicle administration and subsequent naloxone-precipitated withdrawal on response rates are presented in Figure 3. The rats in the vehicle-treated group showed significant reductions in response rates as a function of days ($F_{19,95}=4.74, p=0.0005$). However, significant differences between the vehicle-treated group could not be established on any days ($F_{19,95}$=1.732, p=0.183).

The mean response rate was variable throughout the days of chronic MG administration, as was apparent from a repeated-measures ANOVA conducted on the MG-treated group, which revealed significant differences in the response rates as a function of days ($F_{14,98}=2.68, p=0.002$). While there was no significant difference observed at 12-

, 24-, 36- and 48-hr of post-MG precipitated withdrawal, a withdrawal effect was observed 30 min from precipitation by naloxone (Figure 3). Post hoc tests revealed a significant decrease in response rates of approximately 40% of the baseline levels for the vehicle-treated group ($F_{1,7}=25.255$, $p=0.002$). However, the results showed no significant difference at 12-, 24-, 36- and 48-hr following MG-precipitated withdrawal.

While a significant effect of the 'day' factor was noted for response rates in the morphine-treated group ($F_{19,33}=13.57$, $p=0.0005$), post hoc analyses also revealed a significant reduction in response rates at 30 min post challenge with naloxone ($F_{1,7}=313.84$, $p=0.0005$) compared to the vehicle-treated control group. Subsequently, the response rates remained low, 5% after 30 min withdrawal precipitated by naloxone, increasing slightly to 15% in the first 12-hr after withdrawal was precipitated by naloxone. Post hoc tests also confirmed that this effect was significantly different ($F_{1,7}=89.277$, $p=0.0005$) compared to the vehicle-treated baseline levels. However, no significant difference was observed at 24-, 36- and 48-hr following precipitated withdrawal when compared with the vehicle-treated baseline levels.

Assessment of rimonabant-precipitated MG withdrawal

The effects of 14 days of chronic drug or vehicle administration and subsequent withdrawal precipitated by rimonabant on response rates are illustrated in Figure 4. Vehicle-treated rats showed no significant differences in terms of response rates as a function of days ($F_{19,95}=0.56$, $p=0.927$). Similarly, there was no significant difference on any test days when compared with the vehicle-treated baseline levels.

A repeated-measures ANOVA conducted on the MG group showed a significant difference in response rates as a function of days ($F_{14,98}=3.28$, $p=0.0005$). A withdrawal effect was observed 30 min after rimonabant administration, as the response rate was near the baseline vehicle control at this point in time (see Figure 4). Post hoc tests revealed a significant decrease in response rate when the value was approximately 30% of baseline levels observed for vehicle-treated controls ($F_{1,7}=81.512$, $p=0.0005$). However, the response rates for the remaining 12-hr interval of rimonabant-precipitated MG withdrawal effects were not significantly different from the vehicle-treated baseline levels.

The morphine-treated group showed a significant difference in response rate as a function of days ($F_{14,98}=3.62$, $p<0.001$). However, the significant reduction in rates of responding was only observed 30 min after the challenge with rimonabant ($F_{1,7}=23.700$, $p=0.002$) yielding a reduction that was approximately 48% of the baseline rate of vehicle responding.

Efficacy of MG and buprenorphine to suppress naloxone-precipitated morphine withdrawal

In naïve group of rats, following administration of graded doses of MG (1, 10 and 30 mg/kg, i.p.) to vehicle-treated rats did not significantly change response rates (Figure 5a).

The response rate data on the effects of the graded doses of MG on morphine-dependent rats are presented in Figure 5(b). The MG injection following naloxone-precipitated morphine withdrawal alleviated the intensity of the withdrawal effects observed as significant differences in response rates as a function of dose ($F_{4,20}=7.728$, $p=0.001$).

Post hoc tests revealed significant suppression of response rates for vehicle treatment ($F_{1,5}=29.955$, $p=0.003$) and 1 mg/kg MG ($F_{1,5}=36.906$, $p=0.002$) when compared to response rates from the last day of morphine treatment. However, significant alleviation of withdrawal was observed by MG substitution at 10 and 30 mg/kg MG doses ($F_{1,5}=1.960$, $p=0.220$; $F_{1,5}=0.777$, $p=0.419$), respectively when compared to the response rates from the last day of morphine treatment.

Figure 5(c) illustrates the response rate data on the effects of various doses of buprenorphine on responses by the vehicle-treated rats. Other than an overall effect of dose ($F_{4,28}=15.98$, $p=0.0005$), the response rates after the substitution of the two highest doses of buprenorphine (0.3 and 1.0 mg/kg) were significantly suppressed compared to the last day of morphine treatment ($F_{1,11}=10.026$, $p=0.009$) and ($F_{1,11}=37.464$, $p=0.0005$), respectively.

The effects of graded substitution doses of buprenorphine in morphine-dependent rats are shown in Figure 5(d). Repeated measures ANOVAs revealed a significant difference in response rates as a function of the buprenorphine dose ($F_{4,20}=5.835$, $p=0.003$). Post hoc tests confirmed significant suppression of response rates for the vehicle treatment ($F_{1,5}=29.955$, $p=0.003$) and 0.1 mg/kg buprenorphine ($F_{1,5}=20.534$, $p=0.006$) when compared to the last day of morphine treatment. However, there was a significant alleviation of withdrawal after buprenorphine substitution at 0.3 and 1.0 mg/kg when compared to the last day of morphine treatment ($F_{1,5}=0.906$, $p=0.385$; $F_{1,5}=3.544$, $p=0.118$), respectively.

Discussion

The present study extends the behavioural assessment of MG using a food-maintained operant conditioning task to assess its ability to induce physiological dependence in rats. Previous studies have reported that withdrawal from dependence-producing drugs results in disruption of schedule-controlled behaviour in animals that were maintained on operant schedules of reinforcement (Ford and Balster 1976; Balster 1985). The disruptions of food-maintained operant response rates have been used as effective measures for detecting opioid withdrawal in rats since this procedure involves a highly stable and specific baseline (Ford and Balster 1976; Balster 1985; Schulties et al. 2003). Schedule-controlled behaviour was, therefore, used in this study due to its relative sensitivity in capturing opioid withdrawal based on the response rates of food-maintained behaviour in laboratory animals.

The present study demonstrated that acute administration of MG (5.6, 10, 18, 30 and 50 mg/kg) did not produce a dose-related decrease of food-maintained operant responding in rats. In contrast, consistent with previous studies (Li et al. 2010; An et al. 2012), morphine within the range of doses tested (3, 5.6, 10 and 18 mg/kg), demonstrated a dose-dependent decrease in operant responding maintained under a fixed-ratio schedule. It is interesting to note that various self-reporting studies reported that kratom doses within the range 1 to 15 g (Prozialeck et al. 2012) and 1 to 8 g (Grundmann, 2017) have been used by kratom users but MG content was not determined and reported in these studies. However, Vicknasingam et al. (2010) reported that the estimated dose of MG consumed in humans was within the range of 1.00 - 1.12 mg/kg, while Singh et al. (2014) reported that the MG dose range was around 3.89 - 4.35 mg/kg. These studies were based on self-reported studies where the dose of MG used was based on what the subjects mentioned to the interviewer: i.e., how many glasses of kratom juice they had consumed. The researchers had then

collected samples from the study areas to measure the content of MG. Based on these values, the MG doses used among the kratom users were estimated, while in the present study, the MG doses used (1 – 50 mg/kg) were in the range of 0.16 – 8.06 mg/kg (equivalent of animal doses to human equivalent doses). A previous study had demonstrated that MG up to 10 mg/kg is relatively safe in rats (Sabetghadam et al. 2013). In addition, a dose as high as 806 mg/kg failed to produce any toxic effects in rats (Macko et al. 1972).

It is also interesting to note that in humans, low kratom doses (1 to 5 grams) resulted in reported psychostimulant-like effects while high kratom doses (5 to 15 grams) resulted in opioid-like effects that provided users with pain relief and reduction of opioid-withdrawal symptoms (Prozialeck et al. 2012). It is also worth noting that the amount of kratom consumed by the users were those extracted from raw kratom leaves, indicating that other ingredients (i.e. 7-Hydroxymitragynine (7-HMG)) might abolish the stimulant effects of MG and exert much of their opioid-like effects in high kratom doses. It has been demonstrated that 7-HMG is more potent than MG in exerting opioid-like subjective effects in rats (Harun et al. 2015). In addition, Hemby et al. (2018) also demonstrated a high abuse potential of 7-HMG in rat self-administration studies compared to MG, which exhibited no abuse potential. In addition, the discrepancy in the findings of the current study and those reported in studies with humans may also be attributed in part to the route of administration. Kratom is administered orally by humans, whereas intraperitoneal administration was used in the present study. This difference in route of administration would yield different rates of onset of drug effect, drug levels, duration of effect and metabolism. Another possibility, which would account for MG not producing rate-suppressing effects may be due to the schedule of reinforcement: baseline response rates are generally higher under FR schedule compared to fixed interval (FI) schedules (Varvel et al. 2002). This is supported by a previous report when FI response rates initially ascended, then descended in parallel with increasing doses of nicotine, while FR response rates decreased entirely (Goldberg et al. 1989).

The present study examined whether rats administered MG repeatedly would show behavioural alterations following drug cessation. To the best of our knowledge, this study is the first to examine the ability of MG to induce physiological dependence and withdrawal in rats. Interestingly, this study did not find any marked disruption of operant performance when MG was discontinued, from which the inability of MG to induce spontaneous withdrawal in rats can be inferred. However, this finding needs confirmation from other behavioural models of dependence. To date, studies of humans reported that kratom withdrawal effects were weaker and milder than those observed following discontinuation of opioids (Jansen and Prast 1988; Prozialeck 2016; Henningfield et al. 2018). Typical withdrawal symptoms from kratom abstinence, which include malaise, excessive tearing, aching of muscles and bones and jerky limb movements are more evident in subjects who have been administered a high level of kratom intake (Suwanlert 1975; Ahmad and Aziz, 2012; Singh et al. 2014) and these symptoms were also associated with the dosing and frequency of kratom consumption (Singh et al. 2016; Grundmann 2017; Swogger and Walsh 2018).

The present study was designed to assess disruption of behavior following the administration of naloxone, on the assumption that this would provide a more sensitive measure for mild physiological dependence (Beardsley and Martin 2000). The presence of MG dependence in this study was inferred by the observation that the naloxone challenge produced significant reductions in food-maintained responding compared to pre-drug (baseline vehicle

control) and drug administration condition. The MG withdrawal effect was detected after withdrawal was precipitated by naloxone before the response rate returned to its pre-treatment level within the first 12-hr interval. This was somewhat expected, given that antagonistic challenge to animals treated with dependence-producing drugs typically elicit more intense withdrawal effects than non-precipitated withdrawal (Aceto 1990). The similar finding of a naloxone-precipitated withdrawal effect was observed in morphine-treated rats, which exhibited marked decreases in response rates within 12-hr following the naloxone administration, a finding which coincides with a previous study (Becker et al. 2010). The ability of naloxone to precipitate withdrawal in MG-treated rats confirmed that the MG dosing regimen and the route of MG administration used in this study were successful in inducing MG dependence in rats, although MG elicited only a relatively short-lasting suppression of withdrawal effects (just after withdrawal was precipitated) compared to morphine (within 12-hr after withdrawal was precipitated). Based on these observations, it is conceivable that spontaneous withdrawal effects may be observed if large doses of MG, in excess of 50 mg/kg were administered for prolonged periods. These results parallel findings from those reported with humans, where kratom withdrawal symptoms were much shorter in duration compared to opioids (Singh et al. 2014; Grundmann 2017). In addition, supporting qualitative data from cage-site observation also shows that the magnitude of precipitated withdrawal was greater in morphine-treated rats as indicated by abdominal stretches, limb extension and decreased appetite although these effects were not seen in naloxone-precipitated MG withdrawn rats. The data from vehicle-treated rats also confirmed that the dose of 1 mg/kg naloxone did not alter the responding of the control animals. This strengthens the interpretation that the suppression was more likely due to the precipitated withdrawal effects and not due to the non-specific effects on responding.

The finding that naloxone had precipitated MG withdrawal effects suggested that the μ-opioid receptor is responsible for the development of MG dependence. This is supported by previous studies that reported the pharmacological effects of MG via opioid receptors (Matsumoto et al. 1996; Yusoff et al. 2016; Yusoff et al. 2017). In addition to opioids, a previous report suggested that MG could also possibly act through the same target in the brain as THC (Ismail et al. 2017), suggesting the involvement of cannabinoid receptors in the development of MG dependence (Nanthini et al. 2015). Another highlight of the present study was the finding that rimonabant precipitated a withdrawal effect in both MG- and morphine-treated rats. This finding complements other studies which reported the precipitation of withdrawal effects by rimonabant in morphine-dependent rats (Navarro et al. 1998; Maldonado 2002). The similarity of the precipitated withdrawal effects using rimonabant in both MG and morphine was expected, given the evidence of the shared opioid-like pharmacological properties of the two drugs. These two systems are known to interact between opioids and the endocannabinoid system with various pharmacological effects including antinociceptive action, reinforcing action of drugs and dependence liability (Lichtman et al. 2001; Nanthini et al. 2015). These findings are in agreement with previous studies on the role of the CB-1 receptor in mediating the effects of psychoactive compounds, in which CB-1 receptor knock-out mice failed to acquire self-administration and conditioned place-preference (CPP) to opioids and psychostimulants as well as exhibiting a weaker withdrawal syndrome compared to morphine, cocaine, psychostimulants and THC (Fride 2002; Maldonado et al. 2006). Although more work is needed, these data suggest that cannabinoid receptors may be involved in the development of MG

physiological dependence and, therefore, may represent the neurochemical basis for MG or kratom (i.e. tea, decoction) abuse and dependence liabilities.

The present study extended these findings to determine the efficacy of MG in reducing the severity of morphine withdrawal in rats. The study demonstrated that higher acute doses of MG (10 and 30 mg/kg) may alleviate the suppression of food-maintained lever-pressing responses in naloxone-precipitated morphine withdrawn rats. This study's assessment of MG substitution in an animal model of withdrawal extend the data of Hemby et al. (2018) which demonstrated that MG exposure (over 2-week period) reduced morphine intake using an intravenous self-administration model in rats. In contrast, relatively smaller doses of buprenorphine (0.3 and 1.0 mg/kg) were required to alleviate the suppression of such operant response rates, suggesting it was buprenorphine that produced potent substitution effects, rather than MG. The finding that a single dose of MG effectively blocked morphine withdrawal in rats also coincides with a buprenorphine study (Stoller and Smith 2004). Known as a partial μ-opioid agonist, buprenorphine has a high affinity for, and slow dissociation from μ-opioid receptors in which this mechanism may help to counter the development of morphine physiological dependence (Palma et al. 2015). The findings from the present study suggest that MG also attenuated the morphine withdrawal effects by stimulating μ-opioid receptors through a similar mechanism of action to buprenorphine. This idea is further supported by Kruegel et al. (2016) who demonstrated partial agonist activity of MG at μ-opioid receptors. However, the fact that MG, in contrast to buprenorphine which has abuse liability (Jones et al. 2017) has no or low abuse liability (Harun et al. 2015, Hemby et al. 2018) in alleviating morphine withdrawal effects is interesting and warrants further investigation in order to determine the superiority of MG in relation to buprenorphine as potential pharmacotherapy for OUD. Future studies assessing the relative efficacy of MG with buprenorphine through the self-administration of morphine are warranted to determine the therapeutic potential and relative potency of MG in relation to buprenorphine.

Evidence from other models such as in morphine-withdrawn zebrafish (Khor et al. 2013) also supports the notion that MG may have value as a substituent for the treatment of opioid withdrawal. The current finding that MG can alleviate morphine withdrawal symptoms in rats along with the shared subjective effects between MG and morphine (Harun et al. 2015) further strengthen the therapeutic value of MG as an opioid substitute. However, the present data contradict those reported by Cheaha et al. (2017) in which MG did not decrease the jumping behaviour in naloxone-precipitated morphine withdrawn mice, whereas *M. speciosa* alkaloid extract did. The discrepancy of such findings suggests that some other ingredients of the plant were likely to produce stronger pharmacological effects rather than MG alone (Watanabe et al. 1992; Watanabe et al. 1999). It is worth noting that other factors which may have contributed to the discrepancy of these findings are possibly due to the different methodologies used to assess the outcome of withdrawal. In fact, this speculation is supported by previous reports which concluded that kratom in the form of extracts and decoction was beneficial in reducing an individual's opioid intakes by the alleviation of opioid withdrawal symptoms (Vicknasingam et al. 2010; Grundmann 2017). These findings suggest that further research should focus on the whole kratom leaf rather than just on an isolated compound of MG.

To summarize, the discontinuation of MG was not associated with the disruption of schedule-controlled behaviour in rats. This suggests that MG or analogs might be further investigated as potential therapeutic drugs for treating OUD

and opioid withdrawal. The findings from this study suggest that discontinuation of MG is not associated with overt withdrawal effects, a finding that supports published studies using other behavioural model. For example, Hemby et al. (2018) and Yue et al. (2018) found that MG administration reduced intravenous morphine self-administration in rats but that MG itself did not maintain self-administration. The findings may suggest that MG possesses the desired characteristics of candidate pharmacotherapies for opioid dependence and withdrawal. The successful development of an effective yet less dependence-producing agent from kratom could, therefore, potentially reduce the widespread problem of OUD. However, the value of MG as an opioid management treatment deserves careful scientific consideration to determine its clinical efficacy and safe dosage limits. Indeed, further research, including well-controlled clinical trials will be necessary to confirm these findings as a justification of its use for opioid substitution therapy.

Figure 1: (a) The effects of MG (n=8) and (b) morphine (n=8) on the overall rate of responding of rats in the FR-10 component. The minor segmented area represents the response rate of the vehicle-control session corresponding to the drug group. The major segmented area represents the rate of responding during each session, with drug treatment as expressed as a percentage of the average values during the vehicle-control sessions. Abscissae: drug dose (mg/kg), log scale. * indicates a significant decrease $p<0.05$ in response rates compared to mean rates of responding of the baseline vehicle control (pre-drug treatment).

Figure 2: The effects of MG (n=8), morphine (n=8) and vehicle (n=6) treatments and their withdrawal on the rate of food-maintained responding in rats responding under a fixed-ratio 10 schedule of food presentation. The points shown on the vehicle prior to the treatment day are the control values (i.e. pre-treatment with the vehicle) obtained on the day prior to the start of the daily treatment. Points on days 1-14 represent the values obtained during the 14 days of drug/vehicle treatment. The points shown on day 15 onwards represent the mean responses at every 12-hr interval when the drug treatment was stopped. * indicates a significant decrease $p<0.05$ in response rates compared to the mean rates of responding of the baseline vehicle control (pre-drug treatment).

Figure 3: The effects of MG (n=8), morphine (n=8) and vehicle (n=6) treatment and their withdrawal on the rate of food-maintained responding in rats responding under a fixed-ratio 10 schedule of food presentation. The points shown on the vehicle prior to treatment day are the control values (i.e. pre-treatment with vehicle) obtained on the day prior to the start of the daily treatment. Points on days 1-14 represent the values obtained during the 14 days of drugs/vehicle treatment. The points shown on day 15 onwards represent the values when withdrawal was precipitated by 1 mg/kg naloxone (30 mins of post-naloxone treatment) and at every 12-hr interval of post-naloxone treatment. * indicates a significant decrease $p<0.05$ in response rates compared to the mean rates of responding of the baseline vehicle control (pre-drug treatment).

Figure 4: The effects of MG (n=8), morphine (n=8) and vehicle (n=6) treatment and their withdrawal on the rate of food-maintained responding in rats responding under a fixed-ratio 10 schedule of food presentation. The points shown on the vehicle prior to treatment day are the control values (i.e. pre-treatment with the vehicle) obtained on the day prior to the start of the daily treatment. Points on days 1-14 represent the values obtained during the 14 days of drugs/vehicle treatment. The points shown on day 15 onwards represent the values when withdrawal was precipitated

by 1 mg/kg rimonabant (30 mins of post-rimonabant treatment) and at every 12-hr interval of post-rimonabant treatment. * indicates a significant decrease $p<0.05$ in response rates compared to the mean rates of responding of the baseline vehicle control (pre-drug treatment).

Figure 5: The effects of substitution of various MG and buprenorphine doses on vehicle-treated (5(a) and 5(c)) and morphine-treated rats (5(b) and 5(d)), respectively in the rate of food-maintained responding (withdrawal being precipitated by naloxone) (n=8-12). The points shown prior to treatment with MG and buprenorphine are the control values (i.e. day 14 of morphine dependency) obtained on the day prior to the administration of naloxone (1 mg/kg i.p.). The points shown in between the control values and graded doses of MG and buprenorphine are the values obtained during vehicle substitution. The points following represent the rate of responding during each session with MG and buprenorphine substitution treatment and are expressed as the average of mean responses. * indicates a significant decrease $p<0.05$ in response rates compared to the mean rates of responding occurring on the last day of the morphine treatment (day 14).

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