Mitragynine (Kratom) impairs spatial learning and hippocampal synaptic transmission in rats

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Abstract

Background: Mitragynine is the major alkaloid of *Mitragyna speciosa* (Korth.) or Kratom, a psychoactive plant widely abused in Southeast Asia. While addictive effects of the substance are emerging, adverse cognitive effects of this drug and neuropharmacological actions are insufficiently understood.

Aims: In the present study, we investigated the effects of mitragynine on spatial learning and synaptic transmission in the CA1 region of the hippocampus.

Methods: Male Sprague Dawley rats received daily (for 12 days) training sessions in the Morris water maze, with each session followed by treatment either with mitragynine (1, 5, or 10 mg/kg; intraperitoneally), morphine (5 mg/kg; intraperitoneally) or a vehicle. In the second experiment, we recorded field excitatory postsynaptic potentials in the hippocampal CA1 area in anesthetized rats and assessed the effects of mitragynine on baseline synaptic transmission, paired-pulse facilitation, and long-term potentiation. Gene expression of major memory- and addiction-related genes was investigated and the effects of mitragynine on Ca^{2+} influx was also examined in cultured primary neurons from E16–E18 rats.

Results/outcomes: Escape latency results indicate that animals treated with mitragynine displayed a slower rate of acquisition as compared to their control counterparts. Further, mitragynine treatment significantly reduced the amplitude of baseline (i.e. non-potentiated) field excitatory postsynaptic potentials and resulted in a minor suppression of long-term potentiation in CA1. *Bdnf* and αCaMKII mRNA expressions in the brain were not affected and Ca^{2+} influx elicited by glutamate application was inhibited in neurons pre-treated with mitragynine.

Conclusions/interpretation: These data suggest that high doses of mitragynine (5 and 10 mg/kg) cause memory deficits, possibly via inhibition of Ca^{2+} influx and disruption of hippocampal synaptic transmission and long-term potentiation induction.

Keywords

Mitragynine, Kratom, Morris water maze, field excitatory postsynaptic potentials, long-term potentiation

Introduction

Kratom (or Ketum), also known as *Mitragyna speciosa* (Korth.), has long been used as a psychoactive plant preparation in Southeast Asia. Traditionally, the leaves of *M. speciosa* are reported to be used to treat intestinal infections, muscle pain, as well as to reduce coughing and diarrhea (Chuakul et al., 1995; Jansen and Prast, 1988; Said et al., 1991; Suwanlert, 1975; Vicknasingam et al., 2010; Watanabe et al., 1997). *M. speciosa* preparations have been used by Malay and Thai native people for their opium- and coca-like effects in order to enhance the tolerance of physical labor in high-temperature conditions (Grewal, 1932a,b; Suwanlert, 1975; Tanguay, 2011), as well as an opium substitute in the treatment of opium addiction (Ahmad and Aziz, 2012; Beckett et al., 1965; Burkill, 1935; Burkill and Haniff, 1930; Tanguay, 2011; Vicknasingam et al., 2010; Wray, 1907a,b). The main indole alkaloids present in the young leaves of *M. speciosa* are mitragynine and its analogues, speciogynine, paynantheine, and speciociliatine (Leon et al., 2009; Orio et al., 2012; Ponglux et al., 1994; Shellard et al., 1978a,b). Mitragynine constitutes 66% of the total alkaloid mixture, making it the most abundant active alkaloid derived from the leaves of *M. speciosa* (Chittrakarn et al., 2008; Jansen and Prast, 1988; Shellard, 1974, 1989; Shellard et al., 1978a,b; Takayama et al., 1998).

In humans, chronic consumption of *M. speciosa* preparations is associated with drug abuse and addiction (Hassan et al., 2013; Singh et al., 2014; Suhaimi et al., 2016). Animal studies have shown that the addiction-like effects are mediated by the main alkaloid mitragynine (Yusoff et al., 2016). Mitragynine does not only induce rewarding effects by itself (Yusoff et al., 2016; 2018), but also enhances punishment resistance in natural reward seeking (Ismail et al., 2017). Acute and chronic administrations of mitragynine also reduced locomotor activity in an open field test, impaired passive avoidance and object recognition performance (Apyrani

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et al., 2010; Yusoff et al., 2016). However, other investigators have not detected behavioral impairments following oral mitragynine administration in a Y-maze task designed to assess short-term memory in mice (Hazim et al., 2011). Currently, the effects of systemic mitragynine treatment on spatial learning are unclear.

Senik et al. (2012) investigated the effects of a methanolic *M. speciosa* extract on field excitatory postsynaptic potentials (fEPSPs) and long-term potentiation (LTP) induction in the CA1 field of rat hippocampal slice preparations and reported a profound suppression of non-potentiated fEPSPs, as well as an inhibition of LTP. Further, Ilmie et al. (2015) reported that subchronic oral administration of a standardized methanolic extract of *M. speciosa* improved memory performance during the retention phase of a passive avoidance task, but inhibited LTP induction in the hippocampus CA1 region; though which compound of this extract exerted these effects remains to be determined. Given these conflicting reports and gaps in the literature, we asked whether mitragynine, the major indole alkaloid of *M. speciosa*, can alter spatial learning in rats, as well synaptic transmission and plasticity induction i.e. paired-pulse facilitation (PPF) and long-term potentiation in the hippocampal CA1 field under *in vivo* conditions. Morris water maze testing was conducted to assess spatial learning, which is correlated with hippocampal synaptic activity and plasticity. Here, morphine was used as a positive control based on two reasons. First, because mitragynine acts on the opioid receptor in a similar way to morphine (Kruegel et al., 2016; Matsumoto et al., 2014; Taufik Hidayat et al., 2010), and secondly because of its ability to affect cognitive function in rodents. Several studies have shown that morphine impairs cognition, especially with regard to learning and memory functions (Ilmie et al., 2015; Senik et al., 2012; Yusoff et al., 2016; Zhu et al., 2011). Further, we also measured basal synaptic transmission via input/output (I/O) curves and assessed short-term plasticity via PPF after treatment with mitragynine. PPF is a form of short-term plasticity whereby the fEPSP to a second stimulus is enhanced relative to the first, if the second stimulus is delivered relatively quickly (e.g. <500 ms) after the first (Zucker, 1989). LTP is known as long-lasting, input-specific enhancement of transmission at synapses that results from concurrent, or close to concurrent, activation of both the pre- and postsynaptic neurons (Bennett, 2000; Lynch, 2004). In addition, we also wished to investigate some of the potential cellular and molecular mechanisms, especially intracellular calcium influx and the *Bdnf* and α CaMKII mRNA expression in the brain that could mediate alterations in hippocampal synaptic activity and plasticity induced by acute mitragynine administration.

Experimental procedures

Animals

All experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC), Universiti Sains Malaysia (USM), Pulau Pinang, Malaysia (Approval no.: USM/Animal Ethics Approval/2013/(87)(495). Male Sprague-Dawley rats (aged 8–10 weeks) were obtained from the Animal Research and Service Centre, USM, Penang, Malaysia, weighing between 200–300 g at the time of testing. For all experiments, only new experimentally naïve animals were used. Animals were housed as groups of five rats per cage under

a 12:12-hour light-dark schedule (07:00–19:00) and had free access to food and water.

Mitragynine preparation and treatment

Mitragynine was extracted, isolated, and verified from fresh leaves of *M. speciosa*, as described previously (Utar et al., 2011). Purified mitragynine was confirmed by high performance liquid chromatography (HPLC) and proton nuclear magnetic resonance ($^1\text{H-NMR}$) (400 MHz) analysis (Jamil et al., 2013). Mitragynine obtained by this procedure was approximately 98% pure. For testing, a dose range of 1–10 mg/kg mitragynine was used, with detailed doses adapted for the respective experiments in pre-tests. Mitragynine was dissolved in 20% of Tween 80 as vehicle. The drug injection volume was 1 mL/kg, which was administered intraperitoneally (i.p.) immediately after completion of the daily water maze training trial for 12 days, and before the surgery for electrophysiological recording (single injection). For the Morris water maze experiments, separate groups of animals were treated with either vehicle ($n=12$), morphine (5 mg/kg, $n=12$) or mitragynine (1, 5, or 10 mg/kg, $n=12$ /group; Yusoff et al., 2016). For electrophysiological recordings, vehicle or mitragynine (5 or 10 mg/kg) were used, with doses selected based on the Morris water maze results.

Morris water maze

A circular black pool (diameter, 160 cm) filled with water ($25\pm 1^\circ\text{C}$) to a depth of 70 cm was used for water maze testing. White paint was added to the water to make the platform invisible. This maze was divided into four virtual quadrants and a stainless-steel escape platform (10 cm in diameter) was positioned at the center of one of these quadrants, 2 cm below the water surface. For each trial, an animal was lowered gently into the water, facing the pool wall at defined starting points (north, south, east, west) along the wall of the water maze. The order of the start positions was randomized.

On an initial habituation day, each animal was placed in the pool for a duration of 90 s in the absence of the hidden platform, to provide an estimate of swimming ability and motor control, and to familiarize rats with the test environment and some of the general task requirements (i.e. swimming). Water maze acquisition training commenced on the next day, with the hidden platform placed in the pool. Each animal was trained to navigate toward the hidden platform, with one trial per day for 12 consecutive days. An acquisition trial was terminated when the animal located and climbed onto the platform, or when 90 s had elapsed. In the latter case, the animal was guided and placed on the platform by the experimenter. Once the animal reached the platform, it was allowed to remain on the escape platform for 30 s. Given that rats use spatial extra-maze cues to locate the hidden platform, care was taken that the experimenter always sat at the same position during each trial and test day (Li et al., 2001). The position of the submerged platform was maintained throughout the entire training period.

Twenty-four hours after completion of the last acquisition trial, all animals were given a probe trial (drug-free) in the absence of the platform to examine the retrieval of the previously learned platform position. This probe trial lasted for 90 s and the time spent in each of the four pool quadrants was recorded.

Table 1. List of primers used and their corresponding properties.

Gene	Primers (5'→3')/GC% ^a	Amplicon size (nt)	UPL probe (#)
<i>αCaMKII</i> (alpha calcium/calmodulin-dependent protein kinase II alpha)	F: AAACGAAGGGAGCCATCCT/50 R: TCACGCCATCATTCTCTTG/45	91	49
<i>Bdnf</i> (brain-derived neurotrophic factor)	F: CGGTGGTTTATGTTGACCTGA/48 R: ACAGGACGAAACAGAACGA/50	75	82
<i>Gapdh</i> (glyceraldehyde-3-phosphate dehydrogenase)	F: AATGTATCCGTTGTGGATCTGA/41 R: GCTTCACCACCTTCTTGATGT/48	76	80
<i>Pgk1</i> (phosphoglycerate kinase 1)	F: GATGGGCTTGGACTGTGGTA/55 R: AACAGGACCGTTCCAAACAA/45	88	108
<i>Hmbs</i> (hydroxymethylbilane synthase gene)	F: GTGATGAAGGATGGGCAACT/50 R: TGGTCTCTTGCATGCTATCTG/48	76	109

^aF denotes forward primer whereas R denotes reverse primer.

On the next day, animals were tested for general sensory-motor performance (drug-free) in the presence of a visible platform. The trial lasted for 90 s or until the animal reached the platform and the time to reach the platform was recorded (de Souza Silva et al., 2013).

In vivo electrophysiology

A separate set of animals was used for the electrophysiological studies. Animals were anaesthetized with urethane (2.0 g/kg, i.p., administered as four doses of 0.5 g/kg each, one every 20 min; additional 0.5 g/kg urethane supplements were administered when necessary). After the induction of deep anesthesia, animals were mounted in the stereotaxic frame. In addition, the local analgesic Xylocaine (5 mg/kg) was administered subcutaneously to the skin and tissue along the incision line over the skull. Throughout the experiment, the body temperature was monitored and maintained between 36–37°C by an electrical heating pad and blankets covering the animal.

The skull bone was exposed and small holes were drilled over the following areas (all measurements taken from bregma and the surface of the skull): CA1: AP: –4.2 mm, ML: –3.0 mm, V: –3.0 mm; and CA3: AP: –4.2 mm, ML: +3.0 mm, V: –4.0 mm. A bipolar stimulating electrode (SNE 100, MicroProbes, USA) was placed in the CA3 region to allow stimulation of the Schaffer collateral/commissural pathway (constant current pulses, 0.2 ms pulse duration, pulses delivered at 0.05 Hz). A recording electrode (Perfluoroalkoxy (PFA) Insulated Steel Wire, A-M Systems, USA) was placed in the striatum radiatum of the CA1 region. Screws were placed in the bone overlying the frontal cortex to act as reference and ground connections for the recording electrode. The final ventral placement of the CA3 stimulating and CA1 recording electrodes was adjusted to yield maximum fEPSP amplitude in CA1 in response to CA3 stimulation.

An I/O curve was created by stimulating the CA3 field with increasing intensities (0.1 to 1.0 mA) and the intensity that produced about 50–60% of the maximal fEPSP amplitude in CA1 was used for the subsequent formal data collection (PPF and LTP).

To assess short-term plasticity, PPF was measured by delivering pairs of stimulation pulses (0.2 ms duration) at interstimulus intervals (ISIs) of 20, 50, 100, 200, 500, and 1000 ms.

To examine changes in baseline (non-potentiated) synaptic transmission, baseline (i.e. drug-free) fEPSPs were recorded for

60 min or until a stable baseline was obtained, as determined by examining fEPSPs that were averaged over successive, 10-minute recording epochs. After a stable baseline was established, drug or vehicle was administered (i.p.) and fEPSP recordings continued for three hours after drug administration.

To assess LTP, baseline recordings (same as above) of fEPSPs were followed by delivery of one theta burst stimulation (TBS) of the CA3 region. TBS train was composed of ten stimulus bursts delivered at 5 Hz, with each burst consisting of five pulses at 100 Hz. Recordings of fEPSPs continued for three hours following the delivery of TBS.

All electrophysiological signals were amplified, digitized at 10 kHz by a PowerLab/4SP system (ADInstruments, Australia) and stored using a personal computer and LabChart v.7 software (ADInstruments) for subsequent offline analysis.

Quantitative polymerase chain reaction (qPCR)

In another set of experiments, rats were treated with single administration of mitragynine (30 mg/kg i.p.). This dose was chosen because it is the highest dose that produced significant effects in our previous study (Yusoff et al., 2016). Total RNA was extracted from whole brain tissues of rats treated with 30 mg of mitragynine and a control group ($n=3$ per group) using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. Extracted RNA samples passed both purity and integrity quality controls based on spectrophotometry and gel electrophoresis analysis, respectively. Subsequently, 3 µg of total RNA were converted to complementary DNA (cDNA) prepared in a 20 µL per reaction consisting of 10 mM dNTP mix, 2.5 µM anchored-oligo (dT)₁₈, 20 U Protector RNase Inhibitor, 1X RT buffer and 10 U Transcriptase Reverse using the Transcriptase First Strand cDNA Synthesis Kit (Roche, USA) according to the manufacturer's instructions. Each qPCR reaction was then prepared using 0.001 X synthesized cDNA in a 10 µL reaction consisting of 0.5 µM forward and 0.5 µM reverse primer, 1 X LightCycler 480 Probes Master (Roche, USA) and 250 nM of corresponding Universal ProbeLibrary (UPL) probes (Roche, USA) (see Table 1). All qPCR assays were designed by using UPL Assay Design Centre (www.universalprobelibrary.com) and validated with PCR efficiencies between 98–101% with $R^2>0.990$. The validation step was performed on a serially diluted pooled cDNA synthesized

from the samples described above. All qPCR reactions were cycled in LightCycler 480 Real-Time PCR System using the 96-well plate format. All qPCR reactions were pre-denatured at 95°C for 10 min, followed by 45-cycle of 95°C for 10 s (denaturation), 60°C for 30 s (annealing and elongation) and 72°C for 1 s (signal detection). Fluorescence signals were detected based using the 465 nm and 510 nm excitation and emission filters, respectively. Cycle threshold (Ct) values were determined based on the second derivative maximum method (Luu et al., 2005) and the differences of expression levels between groups were analyzed based on the relative quantification by standard curve approach. Intrastandard normalization of brain-derived neurotrophic factor (*Bdnf*) and alpha calcium/calmodulin-dependent protein kinase II (α CaMKII) (Müller et al., 2016) genes against all the three house-keeping genes, *Gapdh*, *Pgk1*, and *Hmbs* were adopted to calculate the geometric means for statistical testing.

Rat primary neuron culture

Rat embryonic day 16 (E16) to E18 cortical neurons were cultured on glass coverslips coated with poly-D-lysine in Neurobasal medium with B27 supplement (Invitrogen) containing 100 IU/mL penicillin and 100 µg/mL streptomycin. Twenty-four hours after plating, cultures were treated with 1 µM cytarabine for 48 h to remove contaminating glia. Cells were used at 14–16 days for all calcium imaging experiments.

Fura-2 calcium imaging

Intracellular calcium measurement has been described previously (Damodaran et al., 2018). In brief, primary cultured neurons were incubated in the dark for 30 min with 2.5 µM fura-2 acetoxymethyl (Fura-2 AM) (Thermo Fisher Scientific). After being gently washed with normal bath solution containing 140 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂·2H₂O, 1 mM MgCl₂·6H₂O, 10 mM HEPES, 10 mM D-Glucose (pH 7.3), the cells were placed on the Olympus IX81 microscope with the MT-20 illumination unit. Fluorescence was recorded at excitation wavelengths of 340 nm and 380 nm with 300 ms intervals. The Fura-2 ratio of F₃₄₀/F₃₈₀ was calculated as an indicator of intracellular [Ca²⁺]_i. Mitragynine was first dissolved in 100% dimethylsulfoxide (DMSO) to yield a 10 mM solution, and then diluted in bath solution. The neurons were incubated with or without mitragynine for 2.5 min before being bathed in glutamate solution (2 µM L-glutamic acid, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 10 mM HEPES, 10 mM glucose) for 30 seconds. The data were analyzed using Xcellence Rt software (Olympus).

Statistical analysis

All data were expressed as mean ± standard error of the mean (SEM) using Sigma Plot 13.0. Generally, data were analyzed using analysis of variance (ANOVA), followed by Bonferroni post-hoc tests. For the electrophysiological data, the maximum amplitude of the fEPSP was computed offline using LabChart v.7 software (ADInstruments). Amplitude data (mV) were averaged over 10-minute intervals and then normalized by dividing each value by the average baseline (pre-TBS) amplitude of the animal.

For qPCR analysis, the differences between the two groups were tested by using unpaired *t* test with Welch's correction (no assumption of equal standard deviation). The statistical differences were significant at $p < 0.05$.

Results

Mitragynine impairs spatial learning and reference memory in the Morris water maze

Repeated exposure to mitragynine for 12 days (administered each day after completion of the daily training trial) resulted in an impairment in acquisition of an escape response in the Morris water maze task (Figure 1(a)). The escape latencies were significantly prolonged in morphine (5 mg/kg) and mitragynine-treated animals (5 and 10 mg/kg) when compared to the vehicle-treated animals. For morphine-treated animals, escape latency was significantly prolonged on day 5 to day 7 ($p < 0.05$). As for the mitragynine-treated groups, the escape latency was remarkably prolonged in animals that received mitragynine doses 5 and 10 mg/kg. Significant increases in escape latencies were observed on training days 4 to 8. Towards the end of the training trial (day 10–12), all groups reached an asymptotic performance level. However, at the low dose of mitragynine (1 mg/kg), water maze performance did not differ between drug- and vehicle-treated animals ($p > 0.05$). A two-way repeated measure ANOVA conducted on the escape latency data confirmed significant differences due to treatment ($F_{4,51} = 4.430$; $p = 0.004$) and training days ($F_{11,561} = 36.380$; $p < 0.0001$). However, there was no significant interaction between training days and treatment ($F_{44,561} = 1.22$; $p = 0.160$).

Twenty-four hours after the last acquisition trial, all animals were subjected to probe trial (drug-free) testing in order to determine the time spent in the target quadrant (no platform). The time spent in the target quadrant was significantly reduced for animals that had received mitragynine at doses of 5 mg/kg ($p = 0.015$) and 10 mg/kg ($p = 0.0049$) during the previous 12 training days (Figure 1(b)). However, at 1 mg/kg mitragynine, probe trial performance was not impaired compared to rats that had received vehicle treatment during training. In contrast to the effect of mitragynine, animals in the morphine-treated group displayed intact probe-trial performance. It is important to note that drug treatments did not exert any long-term effects on general sensory motor performance, as evidenced by a lack of differences in the escape latencies when animals were required to swim to a visible platform 24 h after the probe trial test (Figure 1(c)).

Mitragynine suppresses non-potentiated fEPSPs in the hippocampal CA1 region

The effect of mitragynine on baseline (non-potentiated) transmission at CA3–CA1 synapses was investigated. For both vehicle- and mitragynine-treated animals (5 mg/kg; injections given after 60 min of drug-free baseline fEPSP recordings), fEPSP amplitude remained relative stable throughout the three-hour period following drug administration (amplitude values in the range of 89–104% of baseline; Figure 2). However, in rats given the high dose (10 mg/kg) of mitragynine, fEPSP amplitude showed a marked decline to about 54% of baseline values, an effect that

Figure 1. The effects of vehicle (Veh), mitragynine (Mit; 1, 5, and 10 mg/kg), or morphine (Mor; 5 mg/kg) on performance in the Morris water maze. (a) Escape latencies (mean $\pm$ standard error of the mean (SEM)) during 12 consecutive training days (one training trial/day). (b) Time spent in the target quadrant during probe trial testing. (c) Escape latencies for the visible platform test (* p <0.05, ** p <0.01, *** p <0.001, vs the vehicle-treated group; n =12/ for all groups).

emerged from the 100th minute to 240 min of the experiment. A two-way ANOVA showed an effect of mitragynine on non-potentiated fEPSP amplitude in the CA1 region, with a significant effect of treatment ($F_{2,216}=92.90$, p <0.0001) and time ($F_{23,216}=5.214$, p <0.0001), as well as a treatment $\times$ time interaction ($F_{46,216}=2.184$, p <0.0001).

These results were confirmed in a separate analysis assessing the effects of mitragynine on fEPSPs recorded during the I/O generation (single 0.2 ms pulses delivered to CA3 at intensities between 0.1–1.0 mA in 0.1 mA increments). Results showed that mitragynine (10 mg/kg) significantly altered I/O curves (Figure 3(a)), with a two-way repeated measure ANOVA revealing significant effects of stimulation intensity ($F_{9,108}=190.266$, p =0.0001) and drug treatment ($F_{2,12}=4.537$, p =0.034), as well as an intensity by drug interaction ($F_{18,108}=2.462$, p =0.0022). The fEPSP amplitude was significantly reduced at the 0.3, 0.4, 0.5, and 0.6 mA stimulation intensities in animals treated with mitragynine 10 mg/kg in comparison to vehicle-treated rats (Figure 3(a)).

Figure 2. The effect of vehicle and mitragynine (Mit; 5 and 10 mg/kg; n =4 for all groups) in the absence of theta burst stimulation to assess changes in baseline (non-potentiated) synaptic strength in the hippocampus CA1 region following drug treatment. Stable baseline field excitatory postsynaptic potentials (fEPSPs) were recorded for 60 min, followed by drug or vehicle administration (intraperitoneally (i.p.); at arrow). Vehicle or a low dose (5 mg/kg) of mitragynine did not result in significant changes in fEPSP amplitude. However, the high dose (10 mg/kg) of mitragynine produced a significant suppression of fEPSPs over the three hours following drug administration. Insets: traces of fEPSPs recorded during baseline and at 240 min. The data are expressed as the mean $\pm$ standard error of the mean (SEM). *** p <0.001 vs the vehicle-treated group at 240 min.

Effects of mitragynine on hippocampal PPF and long-term potentiation

The effect of mitragynine on PPF in the hippocampal CA1 region was investigated. Treatment groups received mitragynine (i.p.; either 5 or 10 mg/kg) and control animals were given 20% Tween 80 (vehicle). In controls, PPF was observed for paired pulses delivered at ISIs between 20–200 ms (Figure 3(b)). Mitragynine did not significantly affect PPF when compared to the control group, with no significant difference between treatment groups ($F_{2,12}=1.313$, p =0.3051) and no treatment by ISI interaction ($F_{10,60}=1.047$, p =0.4168). However, there was a significant effect of ISI ($F_{5,60}=5.112$, p =0.0006), confirming that shorter ISIs were more effective than longer ones in eliciting PPF (Figure 3(b)).

Next, we assessed the effects of mitragynine (5 and 10 mg/kg) on LTP in CA1 induced by TBS applied to CA3. In control animals (20% of Tween 80), the TBS produced sustained LTP, with maximum potentiation of over 100% potentiation and lasting till the end of the experiment (about 60% potentiation at three hours after TBS delivery; Figure 3(c)). Treatment with 10 mg/kg mitragynine resulted in fEPSP amplitude is comparable to that in control animals (Figure 3(c)). However, for animals treated with mitragynine at 5 mg/kg, maximal potentiation reached only about 80% and declined to about 40% at the end of the experiment. Further, levels of potentiation in rats given 5 mg/kg mitragynine

Figure 3. Effects of vehicle (Control) and mitragynine (Mit 5 and 10 mg/kg) administration on field excitatory postsynaptic potentials (fEPSPs) in the hippocampal CA1 region of urethane-anesthetized rats (data are expressed as the mean $\pm$ standard error of the mean (SEM) ($n=5$ /group)). (a) Input-output (I/O) curves generated with increasing stimulation intensities (0.1–1.0 mA) delivered to the contralateral CA3 field. Mitragynine administration (10 mg/kg) resulted in reduced fEPSP amplitude at stimulation intensities between 0.3–0.6 mA. Inserts depict typical fEPSPs elicited by 0.5 and 1.0 mA in rats treated with vehicle (larger amplitude) and 10 mg/kg mitragynine (smaller amplitude) (** $p<0.01$, *** $p<0.001$, vs the vehicle-treated group). (b) Paired-pulse facilitation (PPF) was assessed by applying pairs of stimuli to CA3 at interstimulus intervals ranging from 20–1000 ms and calculating the amplitude ratio of the two fEPSPs elicited in CA1. There were no significant differences among the various treatment groups. Inserts depict typical fEPSPs elicited at an interstimulus interval of 20 ms. (c) Long-term potentiation (LTP) was assessed by recording stable baseline fEPSPs for 60 min, followed by theta burst stimulation (TBS) delivery (immediate after 60 min). Mitragynine administration (5 mg/kg) resulted in a modest suppression of LTP (* $p<0.05$ between Mit 5 and Control). Inserts depict typical fEPSPs recorded at 240 min in a control animal (top) and in rats treated with either Mit 5 (middle) or Mit 10 (bottom). (d) Average fEPSP amplitudes recorded from 121–240 min of the experiment in the three treatment conditions (* $p<0.05$ compared to Control).

and vehicle differed significantly at 70, 220, and 240 min of the experiment (Figure 3(c)). Two-way repeated measure ANOVA revealed a significant time effect ($F_{23,276}=104.665$, $p<0.0001$), but no effect of treatment effect ($F_{2,12}=1.095$, $p=0.3657$). However, there was a significant time $\times$ treatment interaction ($F_{46,276}=1.455$, $p=0.0367$).

In an additional analysis, mean values of the fEPSP amplitude (normalized to pre-TBS baselines) for the last two hours of the experiment (i.e. from two to three hours following LTP induction) were compared among the three experimental groups (Figure 3(d)). This analysis showed that, compared to vehicle-treated rats (fEPSP amplitude at 1.68 ± 0.03 of baseline), LTP levels were significantly lower in mitragynine-treated animals at both the 5 mg/kg (1.49 ± 0.04 ; $p<0.05$) and 10 mg/kg dose (1.48 ± 0.08 ; $p<0.05$).

Effects of mitragynine on expression levels of *Bdnf* and α CaMKII

Quantitative analysis of *Bdnf* (adjusted p value=0.3943) and α CaMKII (adjusted p value=0.8416) gene expression levels showed no significant differences between mitragynine-treated animals and the control groups (Figure 4), suggesting that drug treatment did not alter the gene expression profile for *Bdnf* and α CaMKII in the whole brain samples.

Effects of mitragynine on Ca^{2+} influx

To examine the effects of mitragynine on Ca^{2+} influx, we cultured primary neurons from E16–E18 rats. The neurons received vehicle DMSO or various concentrations of mitragynine treatments for around three minutes, followed by 30 s of 2 μM glutamate. The results from two sample neurons are shown in Figure 5(a) and (b). Treatment of vehicle and mitragynine at various concentrations did not alter $[\text{Ca}^{2+}]_i$. However, pre-treatment with mitragynine at all three concentrations (10, 30, and 100 μM) inhibited Ca^{2+} influx upon glutamate stimulation as compared to the vehicle group (Figure 5(c)). This result demonstrates that mitragynine may impair calcium homeostasis via inhibiting Ca^{2+} influx through glutamate receptors.

Discussion

Kratom is a widely abused drug in Southeast Asia with largely unknown impacts on the cognitive performance of users. Here, we investigated the effects of its main psychoactive ingredient, mitragynine, on spatial learning using the Morris water maze and hippocampal synaptic transmission and plasticity. The water maze task has been extensively used as a tool to evaluate the effects of drugs on spatial learning, which often correlates with activity and plasticity in the septo-hippocampal system (Zheng et al., 2002). In the present study, daily post-training exposure to mitragynine impaired the acquisition of spatial learning in the Morris water maze, as well as memory retrieval during the probe trial. This deficit in spatial learning closely resembles the effects of morphine noted in the present and other studies (McNamara and Skelton, 1992). Interestingly, memory assessed during the probe trial showed that morphine-treated animals were still able to recognize the quadrant where the escape platform was

previously placed, while mitragynine treatment (5 and 10 mg/kg) significantly impaired probe trial performance. Thus, mitragynine appears to produce more severe and long-lasting disruptions of spatial memory formation than those associated with morphine administration. Numerous previous studies have also shown that mitragynine at the dose range tested here does not impair locomotor performance or coordination (Apyani et al., 2010; Yusoff et al., 2016). Thus, learning deficits concluded from behavioral performance in the Morris water maze may not have resulted from impaired swimming performance. In our study, we used the cued learning trial (Vorhees and Williams, 2006) to detect the presence of unspecific factors (i.e. sensory-motor deficits) that might affect the animals' cognitive performance. The cued learning trial showed no significant difference in escape latencies between groups in the presence of the visible platform, which indicates that sensory-motor functions were not compromised in the drug-treated groups (Figure 1(c)).

A study by Zhu et al. (2011) showed that morphine treatment impaired both spatial learning and reference memory. Some studies suggest that spatial working memory during acquisition may not be a crucial step towards forming a reference memory (Bannerman et al., 2008; Niewoehner et al., 2007), which may explain the ability of morphine-treated rats to perform well on the probe trial (present study results). These findings also support the notion that different mechanisms may underlie spatial learning and reference memory formation (Izquierdo et al., 2000).

This is the first report that investigated the effects of mitragynine on baseline (non-potentiated) transmission in the hippocampal CA1 region using fEPSP recordings *in vivo*. We found that after vehicle and low dose mitragynine (5 mg/kg) applications, fEPSP amplitude remained relative consistent throughout the three-hour period following drug treatment. However, mitragynine (10 mg/kg) induced a significant synaptic depression by reducing the fEPSP amplitude to about 54% of baseline values. Thus, it appears that relatively high doses of mitragynine interfere with the hippocampal synaptic transmission by depressing glutamatergic signaling in CA1 neurons (Simon et al., 2001). This conclusion was further supported by analyzing (I/O) curves for fEPSPs elicited by increasing stimulation intensities applied to the contralateral CA3 field (Dunlop et al., 2008). Compared to vehicle-treated animals, I/O curves obtained in the mitragynine group (10 mg/kg) showed a significantly smaller fEPSP amplitude at the stimulation intensity between 0.3–0.6 mA (Figure 3(a)), confirming the effect of mitragynine of disrupting hippocampal synaptic transmission.

PPF is a form of short-term synaptic plasticity elicited by two stimulation pulses delivered at a short ISI (typically <500 ms). At least in part, PPF is thought to be related to increases in transmitter release from presynaptic terminals as a result of Ca^{2+} accumulation following the rapid application of stimulation pulses (Thomson, 2000). Mitragynine application did not result in any significant changes in PPF under the present experimental conditions. Thus, it appears that, in contrast to the pronounced effects of basal synaptic transmission, mitragynine does not exert major effects on this type of short-term synaptic plasticity in the hippocampal CA1 field.

For all electrophysiological experiments, vehicle and mitragynine (5 or 10 mg/kg) were given to the rats i.p. 15–30 min before the start of urethane anesthesia to allow the drug to reach its maximum concentration (C_{max}) during the electrophysiological

Figure 4. (a) Brain-derived neurotrophic factor (*Bdnf*) and (b) alpha calcium-calmodulin-dependent protein kinase II (αCaMKII) gene expression levels in mitragynine-treated (Mit; 30 mg/kg) and control rat whole-brain samples. The data are expressed as the mean $\pm$ standard error of the mean (SEM) ($n=3/\text{group}$).

Figure 5. Ca^{2+} influx elicited by glutamate (Glu) is inhibited in neurons pretreated with mitragynine. (a) Graph of the Fura-2 ratio of a sample neuron treated with vehicle (dimethylsulfoxide (DMSO)) and 2 μM Glu. (b) Graph of the Fura-2 ratio of a sample neuron treated with 10 μM mitragynine, followed by 2 μM Glu. (c) Summary of the Fura-2 ratio changes in neurons receiving Glu application following pre-treatment of either vehicle (DMSO) or various concentrations of mitragynine. *** $p < 0.001$ (vs DMSO), # $p < 0.0001$ (vs DMSO), one-way analysis of variance (ANOVA) followed by Bonferroni post-hoc test.

recordings. The drug was expected to reach its maximum concentration in the body within an hour post-administration. In the literature, there is only a small number of studies on mitragynine pharmacokinetics in rats after oral administration. In most cases, the drug was found to reach C_{max} about 1–2 h after oral administration (20, 40, 50 mg/kg mitragynine) (de Moraes et al., 2009; Janchawee et al., 2007; Parthasarathy et al., 2010). In some instances, perhaps due to its erratic absorption, the plasma concentration over time did not clearly indicate a single time-point of C_{max} , but the drug apparently peaked at one and five hours with approximately similar concentrations, and the declined gradually

over time (Parthasarathy et al., 2010). In our laboratory, we found that the drug reached a peak at one hour post-administration at $\sim 170\text{ng/mL}$ (plasma level) after a single oral dose of 20 mg/kg mitragynine. This is in line with its physicochemical properties, given that mitragynine is lipophilic in nature, acid labile, and of poor water solubility (Ramanathan et al., 2015). In rats, oral administration of this drug resulted in very poor bioavailability (3%) (Parthasarathy et al., 2010). However, its intermediate lipophilicity favored rapid entrance and elimination from inner tissue compartments after intravenous administration. Also, mitragynine, being a psychotropic drug, is expected to show rapid blood

brain barrier (BBB) penetration. Based on the current studies from our laboratory, mitragynine has shown a moderate capacity to accumulate inside brain parenchymal cells (Yusof et al., 2018). In recognition of poor bioavailability following oral administration, an intraperitoneal route of administration was used, which should have resulted in improved bioavailability and a shorter delay to reach C_{max} in plasma.

To analyze the effects of mitragynine on long-term potentiation, we used TBS in the CA3 field to induce LTP of fEPSPs in the hippocampal CA1 area. In control animals, TBS produced over 100% maximal potentiation of fEPSP amplitude, which declined by approximately 30% by the end of the experiment. Animals treated with mitragynine at 10 mg/kg showed levels of LTP that were similar to those seen in the control group. However, there was a statistically significant reduction in fEPSP amplitude averaged over the last two hours of the experiment (i.e. from 61 to 180 min after LTP induction) in rats given 10 mg/kg mitragynine compared to vehicle-treated animals. In the group which received the 5 mg/kg dose, maximal fEPSP amplitude after TBS reached only 70% and LTP gradually declined to about 40% by the end of the experiment. These results suggest that mitragynine may impair long-term synaptic plasticity in the hippocampal CA1 area, even though the effect appears to be modest and should be confirmed in future experiments.

Given the importance of Ca^{2+} influx for the initiation of most forms of LTP and short-term potentiation, we also examined the effects of mitragynine on Ca^{2+} channels in cultured primary neurons from E16–E18 rats. Fluorescent imaging techniques using calcium-sensitive indicators such as Fura-2 are able to monitor changes in postsynaptic calcium levels, such as those seen during LTP induction. Application of glutamate (2 μ M) was found to enhance Ca^{2+} influx in cultured primary neurons (see Figure 5(a)). However, pre-treatment with mitragynine at concentration of 10, 30, and 100 μ M potentially inhibited Ca^{2+} influx upon glutamate stimulation, findings that corroborate previous research. For example, Matsumoto et al. (2005) showed that mitragynine blocks T- and L-type Ca^{2+} channel currents and reduces KCl-induced Ca^{2+} influx in neuroblastoma cells. Based on these results and on our observations of inhibition of glutamate-elicited Ca^{2+} influx, one can speculate that changes in Ca^{2+} signaling underlie the impairments of hippocampal transmission and plasticity, as well as of memory acquisition, seen with mitragynine administration.

The induction of long-lasting, late-phase LTP (L-LTP) is subserved by persistently activated protein kinases and involves changes in gene expression and protein synthesis. Specifically, L-LTP causes activation of cAMP-dependent protein kinase (PKA) and extracellular signal-related protein kinase (ERK) (Impey et al., 1998). Subsequently, several transcription factors such as calcium responsive-element binding protein (CREB) are phosphorylated and activated for the transcription of downstream genes to mediate the changes in the structure and/or function of the synapses. One of the downstream genes is *Bdnf*. Thus, we examined the effect of mitragynine on the expression of *Bdnf*. However, the results of these experiments did not reveal significant changes in *Bdnf* expression induced by the drug. These observations suggest that alternations in *Bdnf* functioning are unlikely to mediate the behavioral and electrophysiological effects noted after mitragynine administration.

LTP induction results in Ca^{2+} influx, triggering intracellular biochemical cascades through activation of alpha calcium-calmodulin-dependent protein kinase II (α CaMKII), a major synaptic protein related to learning and memory in animals and humans (Easton et al., 2013a; Leonard et al., 1999, Lisman et al., 2012). The activation of α CaMKII plays a major role in plasticity induction and maintenance, as well as in behavioral learning (Bliss and Collingridge, 1993) and psychoactive drug use and abuse (Müller et al., 2016). Reinforcing drugs, which induce neuronal activation and LTP, have been shown to enhance α CaMKII activity (Easton et al., 2013a,b; Schöpf et al., 2015). However, this activity increase may be brought about by enhanced mRNA expression and/or phosphorylation (Müller et al., 2016). The results of the present experiments found no changes in the expression of the α CaMKII gene in mitragynine-treated rats. Whether α CaMKII phosphorylation was enhanced still has to be evaluated in future studies. It should also be noted that LTP and memory formation may well be induced in the absence of α CaMKII and its phosphorylation, though at reduced speed (Radwanska et al., 2011; Villers et al., 2014). From that, one may conclude that the inhibitory mitragynine effects on glutamate-induced Ca^{2+} influx may translate to LTP deficits not by changing the α CaMKII or brain-derived neurotrophic factor (BDNF) pathways, but by other, yet to be determined mechanisms. This may, for instance, be brought about by altering the modulatory monoaminergic inputs to the hippocampus originating from the mesencephalon (Yusoff et al., 2016).

In conclusion, our study demonstrates that mitragynine caused profound impairments in spatial memory acquisition and excitatory synaptic transmission in the hippocampal CA1 field, together with a moderate suppression of LTP. Further, mitragynine application resulted in an inhibition of Ca^{2+} influx into neurons, raising the possibility that changes in Ca^{2+} signaling is involved in the behavioral and synaptic impairments elicited by this drug. Thus, mitragynine, particularly at higher doses, has the potential to cause significant cognitive dysfunction and disruption of processing in the hippocampal formation of rodents.

Authors' contributions

ZH, FWS, MAE designed, performed research and analyzed data. ZH, FWS, KHL, MAE, CPM, and HCD wrote the article. All authors read and approved the manuscript.

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