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Assessment of the Discriminative Stimulus Effects and Time Courses of Salvinorin A and Two Synthetic Salvinorin B Derivatives, Methoxylmethyl (MOM) and Ethoxymethyl (EOM), Ethers in Rats Trained To Discriminate U69,593

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ASSESSMENT OF THE DISCRIMINATIVE STIMULUS EFFECTS AND TIME COURSES OF SALVINORIN A AND TWO SYNTHETIC SALVINORIN B DERIVATIVES, METHOXYMETHYL (MOM) AND ETHOXYMETHYL (EOM), ETHERS IN RATS TRAINED TO DISCRMINATE U69,593

by

Lisa M. Bell

A Thesis
Submitted to the
Faculty of The Graduate College
in partial fulfillment of the
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Department of Psychology
Advisor: Lisa Baker, Ph.D.

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ASSESSMENT OF THE DISCRIMINATIVE STIMULUS EFFECTS AND TIME COURSES OF SALVINORIN A AND TWO SYNTHETIC SALVINORIN B DERIVATIVES, METHOXYMETHYL (MOM) AND ETHOXYMETHYL (EOM), ETHERS IN RATS TRAINED TO DISCRMINATE U69,593

Lisa M. Bell, M.A.

Western Michigan University, 2012

Research regarding the psychopharmacology of salvinorin A, the main psychoactive ingredient in the hallucinogenic plant *Salvia divinorum,* has been motivated largely by a recent increase in its recreational use and widespread media attention focused on this plant and its extracts. In addition, there is considerable evidence that drugs acting on kappa opioid receptors (KOR) may have therapeutic potential in the treatment of some neuropsychiatric conditions, including drug dependence and mood disorders. Although the neuropharmacological actions of salvinorin A are well established, only a few studies have explored the behavioral effects of this substance in comparison to the KOR agonist, U-69-593. Salvinorin A appears to have a shorter duration of action *in vivo* than salvinorin B analogues (Wang, Chen, Xu, Lee, Ma, Rawls, Cowan and Liu-Chen, 2008). The aim of current study was to assess the discriminative effects of salvinorin A and two synthetic salvinorin B derivatives, the methoxymethyl (MOM) and ethoxymethyl (EOM) ethers in rats trained to discriminate U69,593. Eight male Sprague-Dawley rats were trained to discriminate U69,593 (0.32 mg/kg, S.C. 30 min) from vehicle in an operant task under a fixed-ratio (FR) 20 schedule of food reinforcement and stimulus generalization tests were conducted with U69,593 (0.02-0.32 mg/kg), salvinorin A (0.06-1.0 mg/kg, I.P.), salvinorin B MOM (0.01-0.6 mg/kg), and salvinorin B EOM (0.005-0.3 mg/kg). Time course tests (30 to 240 min) were also conducted with the highest dose of each test compound.

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I would like to thank Lisa Baker for her patient assistance and ongoing support throughout the research process. Her guidance and mentorship have shaped my life and continue to inspire me. My sincerest gratitude to Amanda Austin, M. Melissa Peet, Larissa Haliw and Sherly Walker for their generosity and assistance during a difficult personal time. Without the help of these women, this research would have come to a halt. I would also like to thank the entire behavioral pharmacology laboratory for their support during the entire research process. The members of this laboratory demonstrate teamwork and compassion on a daily basis. Lastly, I would like to thank the members of my thesis committee: Dr.'s Lisa Baker, Alan Poling and Cynthia Pietras for their guidance and expertise. I am grateful for the research experience which has instilled a love of science and a passion for research in my life. Thank you again to all of those who made this research possible.

Lisa M. Bell

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Introduction

Salvia divinorum (salvia) has been used for its psychoactive effects in Mexico for many centuries (Valdes, Butler, Hatfield, Paul, and Koreeda, 1984) and continues to be used in spiritual and ritual practices as well as recreationally. Salvia is commonly consumed by smoking dried, ground up leaves or through buccal administration and it has a quick onset and brief duration of action (Prisinzano and Rothman, 2008). Salvia's current legal status in the United States is unscheduled, therefore it is unregulated by the U.S. federal government and can be legally grown and purchased. Most states have enacted laws restricting its distribution and use. According to Griffin, Miller and Khey (2008) the legal status of the hallucinogenic plant salvia divinorum is rapidly changing. Legal prohibitions have emerged at the state level. States have enacted legislation proposing to control salvia via different legal mechanisms. Though salvia is unscheduled, it is not approved by the FDA (Food and Drug Administration) for human consumption.

Recreational use of salvia in the United States has increased in recent years (Gonzalez, Riba, Bouso, Gomez-Jabaro and Barbanoj, 2006) , which has prompted considerable research on the behavioral and neuropharmacological effects of this plant and its chemical derivatives. The main psychoactive ingredient in *salvia divinorum*, salvinorin A, is a highly selective kappa opioid receptor (KOR) agonist (Roth, Baner, Westkaemper, Siebert, Rice, Steinberg, Ernsberger and Rothman 2002; Sheffler and Roth, 2003; Chavkin, Sud, Jin, Stewart, Zjawiony, Siebert, Toth, Hufeisen, and Roth, 2004). Salvinorin A is a non-nitrogenous neoclerodane diterpene

(Ortega, 1982) that is structurally dissimilar to all other known psychoactive compounds (Priziano and Rothman, 2008) and is currently the most potent naturally-occurring hallucinogen (Valdes et al., 1984; Siebert, 1994). Thus, in addition to clinical and scientific interests in characterizing the abuse liability of salvinorin A, its unique pharmacological profile may lead to exciting prospects in the development of pharmacotherapeutics for neuropsychiatric disorders (Shippenberg, Chefer, Zapata, and Heidbreder, 2001; Shippenberg, Zapata and Chefer, 2007).

Kappa opioid agonists are a recent focus of drug development research (Prisinzano, Tidgewell, and Harding, 2005), including salvinorin A and its synthetic derivatives. Substantial evidence indicates that KORs modulate brain dopamine levels (Spanagel, Herz, and Shippenberg, 1990). For instance, salvinorin A has been proved to decrease dopamine levels in the caudate putamen, an effect which can be blocked by norbinaltorphimine (norBNI), a selective KOR antagonist (Zhang, Butelman, Schlussman, Ho, and Kreek, 2005). Since psychostimulant abuse and dependence are associated with alterations in dopamine regulation (Wang et al., 2008), the relationship between kappa opioid receptors and dopamine regulation is a potential target for pharmacotherapeutic strategies. Of particular interest, there appears to be a crucial involvement of kappa opioid receptors in modulating some of the abuse-related effects of psychostimulant drugs, including decreased self-administration of cocaine in non-human primates (Prisinzano, et al. 2005). Despite considerable progress in drug abuse treatment research, there are currently no FDA approved pharmacological treatments for psychostimulant abuse that utilize Salvinorin A

(Prisinzano, et al. 2005).

Behavioral pharmacology research of salvinorin A suggests that salvinorin A produces several in vivo effects characteristically mediated by KOR, such as sedation (Fantegrossi, Kugle, Valdes, Koreeda, and Woods, 2005) and depression-like effects (Carlezon, Béguin, DiNieri, Bauman, Richards, Todtenkopf, Rothman, Ma, Lee, and Cohen, 2006). In addition, Salvinorin A has been indicated in causing depressive effects on behavior in animal models, such as decreased locomotor activity (Zhang et al., 2005) and impaired climbing behavior on an inverted screen task (Fantegrossi et al., 2005). Salvinorin A has also been shown to produce antinociception (Ansonoff, Zhang, Czyzyk, Rothman, Stewart, Xu, Zjwiony, Siebert, Yang, Roth, and Pintar, 2006; John, French and Erlichman, 2006; McCurdy, Sufka, Smith, Warnick, and Nieto, 2006) as a result of increased KOR activity.

A few studies have examined the discriminative stimulus effects of salvinorin A and its analogs (Butelman, Harris, and Kreek, 2004; Willmore-Fordham, Krall, McCurdy, and Kinder, 2007; Baker, Killinger, Bell, Peet, Panos, Haliw, and Walker, 2009). These studies have confirmed the importance of kappa opioid receptors in the psychoactive effects of salvinorin A and analogs. The current study implemented drug discrimination procedures to compare salvinorin A with two analogs that are synthetic derivatives of salvinorin B, ethoxymethyl ether (EOM) and methoxymethyl ether (MOM), in rats trained to discriminate U69,593. Drug discrimination procedures are commonly employed by behavioral pharmacologists as a screening tool to investigate the neuropharmacological actions involved in the discriminative

(or "subjective") effects of psychoactive drugs. The current study compared the synthetic derivatives of salvinorin B, EOM and MOM and salvinorin A with respect to potency and duration of action. These compounds were examined in animals trained to discriminate another highly selective kappa agonist, U69,593. Substitution for U69,593 was assessed with a range of doses until complete substitution occurred. Subsequently, tests were administered with a range of post-injection times to examine the duration of action of each kappa agonist.

Methods

Subjects: Eight male, Sprague-Dawley rats acquired from Charles River Laboratories (Portage, MI) were used. The rats were approximately six months old at the beginning of the study. Subjects were fed a restricted diet to maintain reinforcing effects of food reinforcers. Subjects' diets were restricted to once daily feeding of commercial rodent diet, after test/training sessions, to maintain body weights at approximately 85% of free feeding weights. Water was available *ad libitum* in the home cages. Subjects were individually housed in polycarbonate cages with corn cob bedding in a colony with a 12-h light/dark cycle (lights on 0700 to 1900) and a temperature maintained at 20 +/- 2 degrees centigrade and humidity at 50 +/- 5 %. Subjects were maintained according to the general principles of animal husbandry outlined by the National research Council (1996), and all experimental protocols were approved by the Institutional Animal Care and Use Committee of Western Michigan University.

Apparatus: Behavioral training and testing were conducted in eight standard operant conditioning chambers (Med-Associates Inc., Georgia, VT), equipped with three retractable levers on the front panel, a food pellet delivery mechanism located above the center lever, and a 28-V house light located at the top of the rear panel. Lever pressing was reinforced with dustless precision food pellets (45 mg, product # F0021). Experimental events and data collection were controlled using a standard IBM-compatible PC with MED-PC (version 4.0 for Windows) instrumentation and software.

Drugs: Drugs were prepared fresh daily with the appropriate vehicle. Salvinorin A, EOM and MOM (Mailman Research Center, McLean Hospital, Belmont, MA) were prepared fresh daily in a 75% dimethylsulfoxide (DMSO) solution. These compounds were initially dissolved in DMSO and then diluted with sterile water. Salvinorin A doses tested were 0.0625 mg/kg, 0.125 mg/kg, 0.25 mg/kg, 0.5 mg/kg and 1.0 mg/kg. EOM doses tested were 0.005 mg/kg, 0.01 mg/kg, 0.03 mg/kg, 0.1 mg/kg and 0.3 mg/kg. MOM doses tested were 0.01 mg/kg, 0.06 mg/kg, 0.1 mg/kg and 0.6 mg/kg. To produce the appropriate dosing, U69,593 was prepared in sterile water with a few drops of lactic acid. U69,593 doses tested were 0.02 mg/kg, 0.04 mg/kg, 0.08 mg/kg, 0.16 mg/kg and 0.32 mg/kg. All injections were administered at a volume of 1 ml/kg in sterile 1 cc Monoject® syringes. All drugs and vehicle were administered via interperitoneal injection with the exception of U69,593 which was injected subcutaneously. All drug doses were calculated based on the weights of the subjects.

Procedures: Twelve days after the start of food restriction, subjects were acclimated to the test chambers. During an initial 60-minute session, food pellets were delivered on a fixed time 60 sec (FT 60") schedule to familiarize animals with the location and sound of the food source. All levers were retracted during this session. After three days of acclimation, subjects were trained to press a lever to receive food pellets during two to three 20 min. training sessions. Only the center lever was present during these initial training sessions and the animals were reinforced for center lever presses on a fixed ratio 1 (FR 1) schedule.

Once all test subjects were reliably responding on the center lever, training began. Reliable responding on the center lever was achieved within three to six trainning sessions. For drug descrimination training subjects were randomly assigned to the left lever for four animals and the right lever for the other four animals. Animals received either U69,593 or vehicle injections prior to each training session. During the first four training sessions, only one lever was present in each chamber (two sessions with the left lever and two sessions with the right lever). Animals were subcutaneously injected with either U69,593 (0.32 mg/kg) or vehicle 30 minutes prior to training sessions. For half the animals, responses on the left lever were reinforced following drug administration and the responses on the right lever were reinforced following vehicle administration. Conditions were reversed for the four remaining animals. During initial training sessions, responding was reinforced on an FR 1 schedule, which was gradually incremented to an FR 20 schedule. Once animals were responsibly pressing the appropriate lever during preliminary training sessions

with only one lever, both levers were presented to begin drug discrimination training. All subsequent training and testing included the presence of both left and right levers.

U69,593 and vehicle training sessions were alternated to include at least three drug training sessions and at least three vehicle training sessions per week, with no more than two consective sessions under the same stimulus conditions. Discrimination accuracy was determined by the percent of correct lever presses prior to the first food pellet delivery during each training session. The criteria for discrimination acquisition was a minimum of 80% correct responses for at least eight out of ten consecutive training sessions. This criterion was met within 40.25 (± 16.05) training sessions. Once discrimination accuracy was achieved, stimulus generalization tests commenced.

Test sessions were conducted once or twice per week depending on the performance of individual animals in interim training sessions. Training criteria was met by each animal between all test sessions. In these tests, animals received either a novel dose of the training compound or one of the other test compounds. Test sessions were conducted in a similar manner to training sessions with the exception that no reinforcers were delivered and each animal was removed from the operant test chamber immediately following completion of 20 consecutive responses on either lever. Complete stimulus generalization (i.e. drug substitution) was defined as 80% or greater drug-appropriate responding. Training sessions continued to be administered in between test days.

Data analysis: Results of stimulus generalization tests were graphed and depicted in dose-response curves for visual and statistical analyses. The main dependent variables of interest are the percentage of responses made on the drug-appropriate lever and the response rate. Group means and standard errors of the mean were calculated for all doses of each test compound. The group data were statistically analyzed using a repeated measures ANOVA followed by post-hoc comparisons among different doses of each test compound. For test compounds that produced complete stimulus generalization as defined above, a nonlinear regression was conducted on the dose-response curve to determine ED_{50} values.

Results

The dose response functions for all compounds tested are displayed in Figure 1.

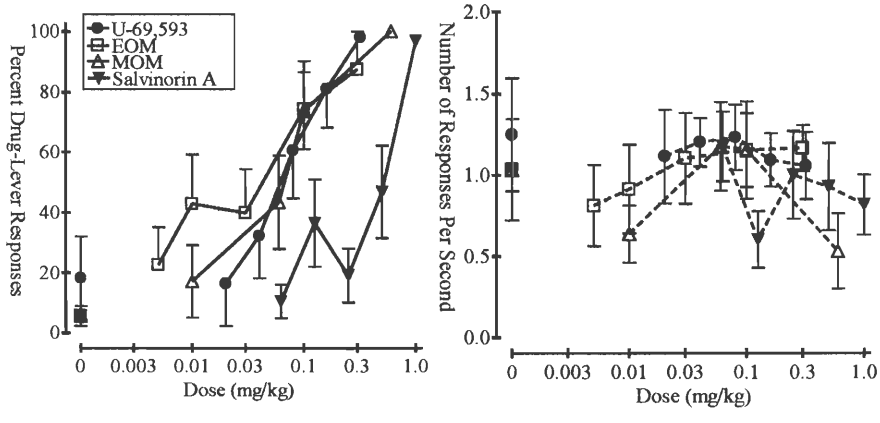

Figure 1. Dose response functions for U69,593 (n=7), salvinorin A (n=8), salvinorin B EOM (n=8), and salvinorin B MOM (n=8).

U69,593 produced dose-dependent increases in drug-lever responding with complete substitution at 0.16 and 0.32 mg/kg. A repeated measures one-way ANOVA on the substitution test results obtained with U69,593 was statistically significant

($F_{5,41} = 6.31$, $p < 0.001$). Dunnett's post-hoc tests showed both 0.16 mg/kg ($p < 0.05$) and 0.32 mg/kg ($p < 0.01$) were significantly different from vehicle.

Salvinorin A and its synthetic derivatives, EOM and MOM all produced complete stimulus generalization to U69,593. Salvinorin A produced dose-dependent increases in U69,593-lever responding and substituted fully at 1.0 mg/kg. A repeated measures one-way ANOVA on the substitution test results obtained with Salvinorin A was statistically significant ($F_{5,47} = 12.44$, $p < 0.001$). Dunnett's post-hoc tests showed both 0.50 mg/kg ($p < 0.05$) and 1.0 mg/kg ($p < 0.01$) were significantly different from vehicle.

EOM and MOM also produced dose-dependent increases in U69,593-lever responding and substituted fully at 0.30 mg/kg and 0.60 mg/kg, respectively. A repeated measures one-way ANOVA on the substitution test results obtained with EOM was statistically significant ($F_{5,47} = 7.64$, $p < 0.001$). Dunnett's post-hoc tests showed both 0.10 mg/kg ($p < 0.01$) and 0.30 mg/kg ($p < 0.01$) were significantly different from vehicle. A repeated measures one-way ANOVA on the substitution test results obtained with MOM was statistically significant ($F_{4,39} = 17.75$, $p < 0.001$). Dunnett's post-hoc tests showed both 0.10 mg/kg ($p < 0.01$) and 0.60 mg/kg ($p < 0.01$) were significantly different from vehicle. Response rates were not statistically significant for any of the compounds tested.

EOM and MOM were of comparable potency to U69,593 and considerably more potent than salvinorin A. A nonlinear regression of the U69-593 dose response function indicated that the ED_{50} was 0.07 mg/kg with 95% confidence intervals 0.02 -

0.29 mg/kg. The ED_{50}s for EOM (0.06 mg/kg, 95% CI: 0.01 - 0.31 mg/kg) and MOM (0.08 mg/kg, 95% CI: 0.05 - 0.12) were comparable to that of U69,593, whereas salvinorin A ED_{50} was considerably higher (0.72 mg/kg, 95% CI: 0.08 - 6.2 mg/kg).

Following the completion of dose response tests, the highest dose of salvinorin A, EOM and MOM were examined at different post-injection intervals. Figure 2 depicts the results of time course tests administered with salvinorin A (1.0 mg/kg), EOM (0.30 mg/kg) and MOM (0.60 mg/kg).

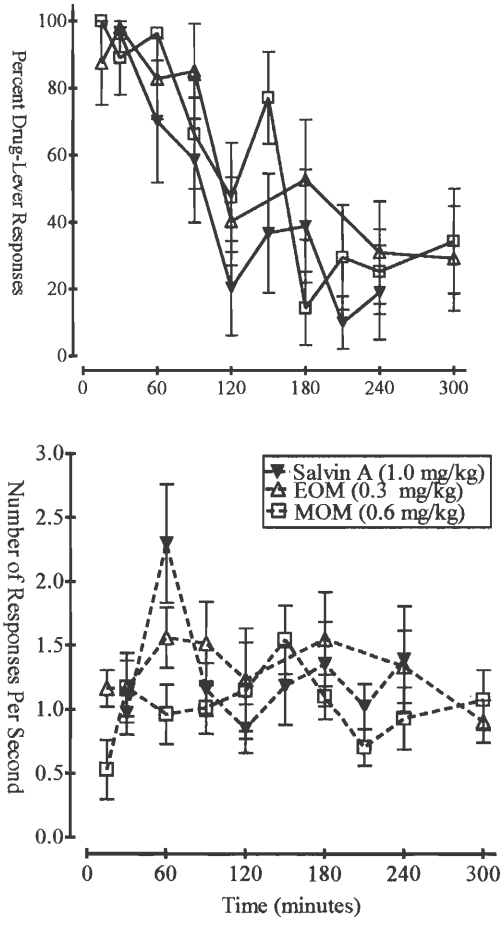

Figure 2. Time Course of Salvinorin A, EOM, and MOM in animals trained to discriminate U69-593 (n=8).

The time course functions of these compounds were relatively similar, although the duration of action of EOM and MOM appeared to be slightly longer than that of salvinorin A. EOM and MOM were discriminated by most animals 60 minutes after administration, whereas salvinorin A discrimination was below 80% 60 minutes after injection.

A repeated measures one-way ANOVA on the time trial test results obtained with salvinorin A was statistically significant ($F_{7,55} = 2.59$, $p < 0.01$). Dunnett's post-hoc tests showed that 120 minutes ($p < 0.01$), 150 minutes ($p < 0.05$), 180 minutes ($p < 0.05$), 210 minutes ($p < 0.01$) and 240 minutes ($p < 0.01$) post injection times were significantly different from 30 minutes post injection time. Results obtained at 60 minutes and 90 minutes post injection were not statistically significant in comparison to 30 minutes post injection. A repeated measures one-way ANOVA on the response rates were statistically significant ($F_{7,55} = 3.36$, $p < 0.0144$). Dunnett's post-hoc tests showed that results for 60 minutes post injection ($p < 0.01$) were significantly different from the 30 minutes post injection time. Response rates at all other post injection times were not statistically significant for salvinorin A time trials.

A repeated measures one-way ANOVA on the time trial test results obtained with EOM was statistically significant ($F_{7,55} = 5.46$, $p < 0.01$). Dunnett's post-hoc tests showed that 120 minutes ($p < 0.05$), 240 minutes ($p < 0.01$), and 300 minutes ($p < 0.01$) post injection times were significantly different from 15 minutes post injection time. Results obtained at 30 minutes, 60 minutes, 90 minutes and 180

minutes post injection were not statistically significant. Response rates were not statistically significant for EOM time trials.

A repeated measures one-way ANOVA on the time trial test results obtained with MOM was statistically significant ($F_{9,79} = 6.14$, $p < 0.01$). Dunnett's post-hoc tests showed that 180 minutes ($p < 0.01$), 210 minutes ($p < 0.01$), 240 minutes ($p < 0.01$) and 300 minutes ($p < 0.01$) post injection times were significantly different from 15 minutes post injection time. Results obtained at all other post injection times (30 minutes through 150 minutes) were not statistically significant in comparison to 15 minutes post injection. A repeated measures one-way ANOVA on the response rates were statistically significant ($F_{9,79} = 2.29$, $p < 0.0273$). Dunnett's post-hoc tests showed that results for 150 minutes post injection ($p < 0.01$) were significantly different from the 15 minutes post injection time. Response rates at all other post injection times were not statistically significant for MOM time trials.

Discussion

The present findings are consistent with previous reports indicating kappa opioid receptor involvement in the discriminative stimulus effects of salvinorin A (Butelman et al., 2004; Willmore-Fordham et al., 2007; Baker et al., 2009). In addition, the results extend these findings to lower doses of U69,593 and salvinorin A. Willmore-Fordham et al. (2007) trained male Sprague-Dawley rats to discriminate a dose of 0.56 mg/kg U69,593 from vehicle. At all doses tested (1.0, 1.9, 3.0 mg/kg), salvinorin A exhibited full substitution for U69,593 and these effects were blocked by the kappa receptor antagonist, nor-BNI. The results of these drug discrimination

investigations support the idea that salvinorin A produces effects similar to those of U69,593. In addition, these effects can be modulated by the administration of a selective opioid antagonist. Together, these findings provide insight into the neuropharmacological mechanisms of salvinorin A, which can be compared in the current study to the salvinorin B ethers tested.

Butelman et al. (2004) assessed the effects of salvinorin A in rhesus monkeys trained to discriminate the kappa agonist U69,593. Ketamine was also tested to rule out the possibility that animals might simply respond similarly to another hallucinogen. These investigators conducted cumulative dosing procedures and time course tests with pretreatment periods ranging from 5 to 120 minutes. Results demonstrated that salvinorin A and U69,593 produced similar dose and time-dependent functions, suggesting similar properties of these two drugs. The current results are consistent with the findings discussed above and show similar dose-dependent response functions for salvinorin A and U69,593. Furthermore, the current study extends these findings to the salvinorin B synthetic derivatives, the EOM and MOM ethers. Butelman et al. (2004) also tested the kappa antagonists, quadazocine and GNTI in combination with salvinorin A. Both antagonists blocked the substitution of salvinorin A, although GNTI effectively blocked salvinorin A substitution in only two of the three subjects tested. These findings support that the mechanism of action occurs at the kappa opioid receptor sites. Although the current study did not evaluate the effects of kappa antagonists on salvinorin A discrimination, future studies that assess these combinations are warranted.

Braida, Limonta, Pegorini, Zani, Guerini-Rocco, Gori, and Sala (2007) assessed salvinorin A's effects on swimming and conditioned place preference in zebrafish. In the study by Braida et al. (2007), fish were observed for 30 seconds following injection and for 30 second intervals every 5 minutes over a 30 minute period. Swimming behavior was scored according to an operationally defined rating scale. Dose-dependent effects were observed in the swimming task. Lower doses of salvinorin A accelerated swimming behavior, whereas higher doses tended to slow swimming behaviors. No effect was observed at moderate doses. Only one of the lower doses produced hallucinogenic-like effects in swimming behavior (a score of 8 meaning frenetic swimming). Dose-dependent effects were also observed in the conditioned place preference task. Subjects injected with lower doses of salvinorin A preferred the side of the tank in which they had been placed following drug administration, indicating a preference for that side and suggesting that salvinorin A might have reinforcing effects. However, at higher administered doses, evidence for conditioned place aversion was observed. This information could be critical to studies of the therapeutic properties of salvinorin A, particularly those studies that are concerned with drug abuse therapy. Though research is thus far limited, salvinorin A has shown potential in treating stimulant abuse (Roth et al., 20005) through regulation of dopamine levels in the brain (Butelman et al., 2004.) The current study suggests that drug abuse therapies using salvinorin A could be expanded to include the examination of salvinorin B derivatives, such as those shown in the current study to have similar dose and time-dependent functions to salvinorin A.

Future studies may assist in the development and screening of salvinorin A analogs for potential pharmacotherapy. Future investigations of these analogs and related compounds may assist in the development of potential pharmacotherapeutic agents targeting the KOR/dynorphin system. Given the recent attention on the potential abuse of this substance, further investigation into the discriminative stimulus effects of salvinorin A and its synthetic analogues are warranted.

WESTERN MICHIGAN UNIVERSITY

Institutional Animal Care and Use Committee

Centennial
1903-2003 Celebration

Date: November 12, 2008

To: Lisa Baker, Principal Investigator

From: Robert Eversole, Chair

Re: IACUC Protocol No. 08-10-02

Your protocol entitled "Behavioral Pharmacology of Salvinorin A and its Chemical Derivatives" has received approval from the Institutional Animal Care and Use Committee. The conditions and duration of this approval are specified in the Policies of Western Michigan University. You may now begin to implement the research as described in the application.

The Board wishes you success in the pursuit of your research goals.

Approval Termination: November 12, 2009

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