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The Kappa Opioid Agonist, Salvinorin A, Attenuates Locomotor Effects of Morphine but not Morphine-Induced Conditioned Place Preference

Stacy Dianne Engebretson

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by

Stacy Dianne Engebretson

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WE HEREBY APPROVE THE THESIS SUBMITTED BY

Stacy Dianne Engebretson

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Psychology
(Department)

Lisa E. Baker, Ph.D.
Thesis Committee Chair

Behavior Analysis
(Program)

Alan D. Poling, Ph.D.
Thesis Committee Member

Cynthia J. Pietras, Ph.D.
Thesis Committee Member

APPROVED

Dean of The Graduate College

Date June 2012

THE KAPPA OPIOID AGONIST, SALVINORIN A, ATTENUATES LOCOMOTOR EFFECTS OF MORPHINE BUT NOT MORPHINE-INDUCED CONDITIONED PLACE PREFERENCE

Stacy Dianne Engebretson, M.A.

Western Michigan University, 2012

Salvinorin A (SA), a selective kappa opioid receptor agonist, is the main psychoactive ingredient in the plant *Salvia divinorum*. The addiction potential of this naturally occurring hallucinogen is currently under investigation using well-validated preclinical screening procedures, including conditioned place preference (CPP). The primary aim of the current study was to determine the effects of SA on CPP established by morphine in adult rats. A secondary aim was to determine if the vehicle used to dissolve SA, dimethylsulfoxide (DMSO), influenced the outcome of SA place conditioning. Rats were randomly assigned to one of four treatment groups: morphine (10 mg/kg) vs. saline; SA (0.4 mg/kg) + morphine (10 mg/kg) vs. DMSO + saline; DMSO vs. saline; SA (0.4 mg/kg) vs. DMSO. Rats were exposed to a 15-min habituation session for three consecutive days. Daily 30-min place conditioning trials were conducted over the next eight days followed by a test day. Morphine initially suppressed locomotor activity but activity increased with repeated exposure to morphine, whereas the combination of SA and morphine reduced morphine-induced locomotor activity. However, SA did not attenuate morphine-induced CPP. In a second experiment, CPP trials were conducted with SA prepared in either a DMSO-water (3:1) solution or in an ethanol/TWEEN80 /water (1:1:8) solution. Regardless of the vehicle used, results of experiment 2 indicated that SA failed to establish CPP and produced modest aversive effects.

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Stacy Dianne Engebretson

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INTRODUCTION

Salvinorin A is a relatively new hallucinogenic drug that acts as an agonist at the kappa-opioid receptor. Derivatives of this compound are currently under investigation for potential therapeutic use. Opioid receptor agonists have been traditionally used in the development of novel pharmacotherapeutics for pain management, mood disorders and substance dependence (Prisinzano, Tidgewell, and Harding, 2005; Hasebe et al., 2004; Carr and Lucki, 2010). By studying the pyschoactive properties of kappa-opioid receptor (KOR) agonists, such as derivatives of salvinorin A, a better use of antagonists may be developed to help treat patients with psychological disorders whose symptoms resemble those seen with KOR activation. For instance, KOR agonists produce disruptions in cognition and perception that resemble some of the symptoms of schizophrenia; these effects can be blocked using KOR antagonists (Nemeth et al., 2010) and the schizophrenic-like symptoms are resolved. Furthermore, there is some evidence to suggest salvinorin A, as well as other k-opioid agonists, may have anti-depressant effects (Hanes, 2001; Braida, 2009), inspiring prospective use of KOR agonists in the development of anti-depressant medication. Alternate therapeutic uses of KOR agonists may be for opioid or psychostimuluant addiction rehabilitation. Previous research has examined the attenuation effects of synthetic KOR agonists on the conditioned rewarding effects of opioids (Huang et al., 2008) and psychostimulants (Tomasiewicz et al., 2008). Preliminary research evaluating the ability of KOR agonists to attenuate the reinforcing effects of other drugs of abuse is still ongoing.

In understanding the potential for salvinorin A to be used in such therapies, it is imperative for public safety that the risk for salvinorin A addiction be assessed using validated behavioral assays. One validated method for studying addiction potential is to explore the inherent rewarding effect of a drug through conditioned place preference (CPP). CPP repeatedly pairs a drug with a neutral

environment. If the drug in question successfully converts the environment from a neutral stimulus to a conditioned stimulus, then the environment will elicit a conditioned rewarding effect as measured by increased time spent in that space compared to control. More research is needed to conclude whether salvinorin A, by means of CPP, may function as a conditioned reinforcer. The present study utilized the CPP paradigm to determine if salvinorin A is capable of establishing a conditioned place preference. In addition, the present study also evaluated salvinorin A's ability to attenuate the conditioned rewarding effects of morphine.

Origin and History of Salvia Divinorum

Salvia divinorum, or diviner's sage, is a species of *Salvia* found in the mountains of Oaxaca, Mexico (Valdés III, 1994). *Salvia* is a genus of plants in the mint family commonly known as "sage"; its name is derived from the Latin *salvus,* "to save," due to its alleged healing properties (USDA, 2012; Colins English Dictionary, 2012). Native tribal healers, Mazatec curanderos, use *Salvia divinorum* as an aid in spiritual rituals to commune with God (Valdés III, 1994) and to treat a variety of ailments including diarrhea, headaches, rheumatism and alcoholism (Prisinzano, 2005; Valdés III, 1984). *Salvia divinorum* grows in clones that extend over one meter high with blossoms of white corolla and purple calyces above hollow square stems (Valdés III et al., 1983). Its defining features are flowers, which makes classification difficult when they are not in bloom (Valdés III, 1994). After successful identification, *Salvia divinorum* specimens were brought to the United States and Alredo Ortega dubbed its main psychoactive ingredient as *Salvinorin A*. Later, salvinorin A was isolated by Leander Valdés (Valdés III et al., 1984). Other extractions, Salvinorin B through F, were also isolated by Valdes but were not shown to produce psychoactive effects (Valdés III, 1994). More details on the chemical structure and variations of salvinorin A are discussed below.

Recreational and ritual use of *Salvia divinorum* or its main ingredient, salvinorin A, varies depending on the preference of the user. People may drink a tea-like infusion, chew on a quid of leaves, crush the leaves and ingest the resulting juice, smoke the leaves or inhale a vapor derived

from heating a leaf's extracts, salvinorin A. Mazatec curanderos traditionally will collect fresh leaves and crush the leaves finely and infuse them in water, making a bitter-tasting tea, or sometimes will have patients eat the leaves (Valdés III, 1994). If preparing salvinorin A for communion with spirits, curanderos have found that infusions incorporating fewer than 20 pairs of leaves do not adequately produce desired, hallucinogenic visions. For satisfactory hallucinogenic experiences, curanderos suggest that infusions require 50 – 100 leaves (Valdés III et al., 1983). Due to the large requirement of fresh leaves for hallucinations and the bitter-tasting infusions, original investigators speculated that salvinorin A had a low abuse liability. However, recreational users in Mexico and USA smoke dried leaves, which eliminates the effort of collecting a large number of fresh leaves (Valdés III, 1994). Moreover, less bitter strains of the plant have been discovered and were cloned for recreational use in the United States (Valdés III, 1994).

The potency of salvinorin A was originally assessed to be similar to that of mescaline based on open field testing in rodents, generalizing that human doses would range from 0.2 – 0.6 mg/kg (Valdés III, 1994). However evidence from Siebert (1994) found that salvinorin A potency in humans may be greater than suspected. In a series of experiments, Siebert (1994) investigated discernible psychoactive effects of salvinorin A through various routes of administration with human participants. He reported that if participants swallowed leaves (dosing equivalent of 10 mg), salvinorin A was essentially inactive. A solution of 2 mg salvinorin A dissolved in 1 ml anhydrous ethyl alcohol sprayed directly onto the lining of the mouth was reported to be a more effective route of administration than swallowing leaves, though the salvinorin A-ethyl alcohol spray did not produce reliable nor predictable effects, presumably because saliva rinsed away a majority of the applied salvinorin A. Siebert (1994) found the most dependable route to produce definite psychoactive effects was to have participants chew and suck on leaves. Moreover, chewing and sucking on 4-5 pairs of leaves similar to a coca-leaf quid maintained stronger visions which lasted a longer period compared to other routes (Siebert, 1994; Valdés III, 1994).

A common recreational route of administering salvinorin A is to smoke dried *Salvia divinorum* leaves yielding hallucinations in five to six inhalations (Valdés III, 1994). When inhaled, the effects of salvinorin A have been characterized as an intense hallucination lasting from a few minutes to up to two hours (Valdés III, 1994). Heating salvinorin A and inhalation of the vapors has created the most potent hallucinogenic experience, with effective doses ranging from 200-1000 μg (Valdés III, 1994). With human volunteers, Siebert (1994) heated 200 -500 μg of salvinorin A extracted from *Salvia divinorum* leaves on a piece of foil and had participants inhale the vapors through a tube. The resulting hallucinogenic experiences lasted anywhere from 30 minutes to two hours, with a threshold of psychotropic effects at a dose of 200 μg. However, doses over 500 μg produced delirium and required careful monitoring by the moderator (Siebert, 1994). In fact, regardless of how salvinorin A was prepared, the reported hallucinogenic effects in humans last from 30 minutes up to two hours, with the peak effects occurring approximately one hour after administration. Currently salvinorin A is the most potent naturally occurring hallucinogen (Roth et al., 2002).

Pharmacology of Salvinorin A

The most abundant of source salvinorin A can be found within the resin of *Salvia divinorum*'s leaves; especially potent resin resides within the subcuticular space of a leaf's grandular trichome (tiny hair-like protrusions; Siebert, 2004). Once extracted, unlike other known hallucinogens (i.e., *N, N*-dimethyltryptamine, psilocybin, mescaline, lysergic acid diethylamide, or ketamine), salvinorin A does not contain a nitrogen atom and thus is not categorized as an alkaloid (Roth et al., 2002). Instead, salvinorin A is considered a neoclerodane diterpene, a bicyclic organic compound composed of four isoprene units. In fact, it is the first documented diterpene hallucinogen (Valdés III, 1994). The unique molecular arrangement of salvinorin A may be the core to its psychoactive effects. Extracts from another member of the *Salvia* family, *Salvia splenden*, has a similar geometric figure to salvinorin A. This extract, known as Splendin, has also been reported to have some pyschoactive effects (Roth et al., 2002).

Salvinorin A's pharmacological target was first profiled by Roth et al. (2002) using cloned human G protein-coupled receptors (GPCR) that contained a range of 48 molecular targets including receptors, transporters and ion channels. After bathing the GPCR proteins with salvinorin A, chemicals known to bind to individual receptors were applied. If the binding of a ligand was inhibited, this indicated that salvinorin A had successfully attached to a particular receptor site. Due to the known affinity of the applied ligands, the affinity of salvinorin A at specific receptors was also quantified.

Results of Roth's et al. (2002) receptor binding studies indicate that salvinorin A is a very potent and selective KOR agonist. Although salvinorin A did bind at mu and delta opioid receptors (MOR and DOR, respectively) it did not do so at any significant level. Chavkin et al. (2004) also evaluated the potency of salvinorin A using radioligand binding, functional studies and through measuring conductance of G protein-gated K+ channels. Their results confirm the original conclusion of Roth et al. (2002) that salvinorin A is a potent, full KOR agonist. Similar to other KOR agonists, in vivo assays have previously found salvinorin A to produce sedation and decreased motor coordination in mice during inverted screen tasks (Fantegrossi et al., 2005), produce antinociceptive effects in mice in tail flick and hot plate tests of nociception (John et al., 2006; McCurdy et al. 2006), and increase immobility and decrease swimming behaviors of rats in a forced swim test (Carlezon et al., 2006). In nonhuman drug discrimination studies, salvinorin A substitutes for the discriminative stimulus effects of synthetic KOR agonists like bremazocine, U-69593, and U-50488 (Butelman et al., 2010; Baker et al., 2009) and produces distinct discriminative stimulus effects from MOR and delta opioid receptor (DOR) agonists (Butelman et al., 2010), as well as other hallucinogens (Butelman et al., 2010; Killinger et al., 2010; Peet and Baker, 2011). Braida et al. (2008) found salvinorin A-maintained lever pressing was blocked by delivery of a cannabinoid receptor antagonist, suggesting that salvinorin A's effects are mediated through endocannabanoid system. However, Walentiny (2010) proposed that cannabanoid activation at KORs, not salvinorin A activation of

endocannabanoid receptors, may be responsible for Braida's et al. (2008) findings that cannabanoid receptor antagonists attenuated salvinorin A-self administration.

Pharmacology of Morphine

Morphine is an active alkaloid isolated from opium, the dried sap found in the unripe seed pods of *Paper somniferum*. Its molecular target is the opioid receptor. Opioid receptors are G-protein coupled receptors commonly classified as one of three different subtypes, KOR, MOR and DOR. Activation of KOR, MOR, or DOR elicits analgesic effects, whereas psychotomimetic effects are only obtained via activation of KOR and euphoric effects occur via MOR activation. One suggestion is that the opposite effects of opioid receptors are due to arrangement of receptors and anatomical position within the brain (Carlezan and Miczek, 2010).

Conditioned Place Preference

Conditioned place preference (CPP) utilizes Pavlovian conditioning principles to associate an unconditioned stimulus (drug treatment) with a set of contextual stimuli (test chamber). Subjects are exposed to an unconditioned stimulus (e.g. drug) just prior to being placed into a neutral environment. The neutral stimulus (e.g. the environment) is associated with the unconditioned response to the drug (e.g. reward or aversion) and the neutral stimulus becomes a conditioned stimulus. As a conditioned stimulus, the environment will elicit a conditioned response associated with the unconditioned effects of the drug (reward or aversion). If the drug produced reward, then the conditioned environment will produce conditioned reward. The conditioned reward is measured by a subject's preference for the drug-paired compartment compared to the vehicle-paired compartment when the animal is in a drug-free state. If the drug produced aversion, then the conditioned environment will produce conditioned aversion. The conditioned aversion is measured by a subject's avoidance for the drug-paired compartment compared to the vehicle-paired compartment when the animal is in a drug-free state.

During CPP, a contiguous association is established; an immediate temporal contiguity exists between the unconditioned and conditioned stimuli. Known reinforcers such as food (Spyraki et al., 1982; Perks and Clifton, 1997) and water (Perks and Clifton, 1997), as well as some novel stimuli, will establish place preferences (Bevins and Bardo, 1999). There is also a strong concordance between drugs that establish CPP and reinforce self-administration of drug, including psychostimulants, opioids and ethanol (as cited in table 1 of Bardo and Bevins, 2000; as cited in Tzschentke, 1988).

There are a few limitations to the CPP paradigm. As Bardo and Bevins (2000) identify, CPP may induce novelty-seeking on test days, although adequate exposure in both drug and vehicle environments via habituation prior to conditioning trial, or providing a novel environment on test day to serve as a novelty control should counteract this. Another limitation is the individual's initial preference for one context over another; the two methodologies to reduce this preference have their own problems. If one pairs the drug with the preferred context, a ceiling effect may develop in assessing the extent of "preference" of an environment. If one pairs the drug with the non-preferred context, the drug may be reducing an aversion instead of establishing preference. Other limitations include the inability to determine a dose-effect curve within a single animal, requiring the use of a large number of subjects for between subject comparisons.

However, Bardo and Bevins (2000) note several advantages to using CPP in preclinical drug screening. It is usually sensitive to low doses, can sometimes be obtained using only a single drug pairing, measures both reward and aversion, can be tested when the animal is in a drug free state, does not require surgeries, CPP procedures typically yield a monophasic dose-effect curve in contrast to the inverted U-shaped dose-effect curve produced by self-administration and finally CPP can assess locomotor activity along with conditioned reward/aversion. Assessing locomotor activity is an advantage to the CPP paradigm because there common relationship between the neural mechanisms of drug-reward and locomotor activity and can be used as an additional dependent measure or in correlation with conditioned place preference. CPP is also directly sensitive to

alterations in motivational state, changes in reinforcer value, as well as taste aversion (Perks and Clifton, 1997). Katz and Gormezan (1979) note that CPP is a rapid and fairly inexpensive procedure to measure drug-induced motivational processes.

Kappa and Mu Opioid Receptor Involvement in Reward Processing

MOR activation indirectly disinhibits secondary and tertiary dopaminergic neurons within the ventral tegmental area (VTA; Margolis et al., 2003; Johnson and Roth, 1992). Figure 1 illustrates the interaction between the GABAergic and dopaminergic neurons within the VTA in relation to MOR or

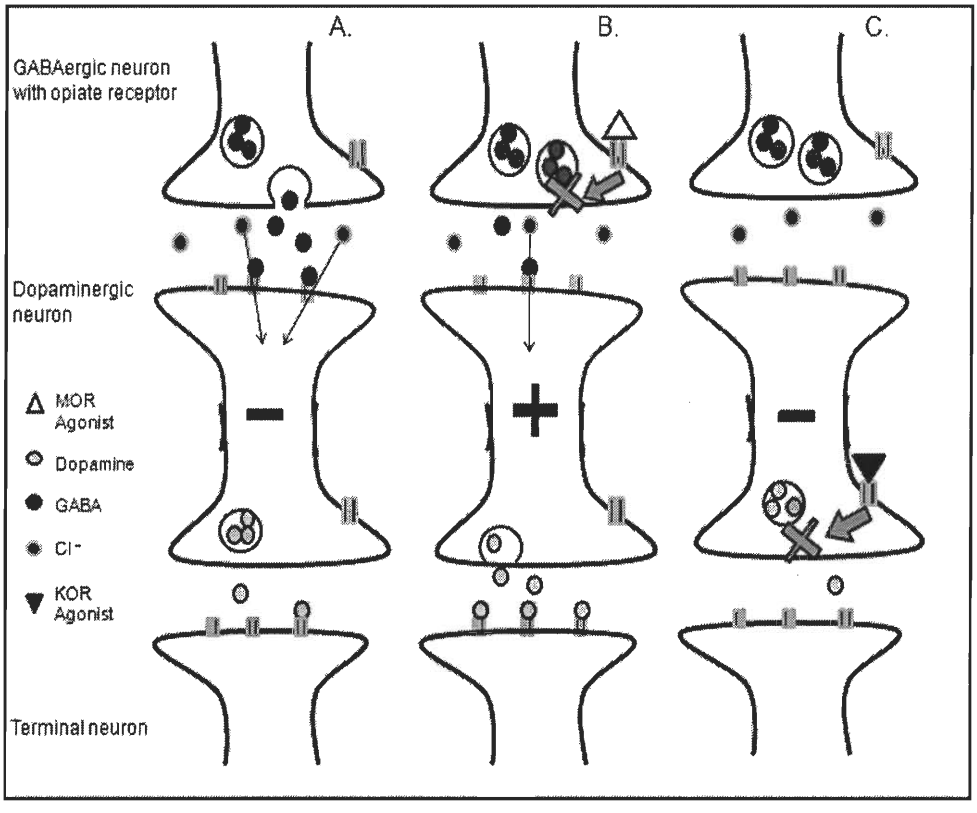

Figure 1. Activation of mu- and kappa-opiate receptors on dopaminergic neurons. A) Without the activation of opioid receptors. B) The mu-opiate receptor disinhibitory effect on dopaminergic neurons. C) Kappa-opioid receptors direct inhibitory effect on dopamine release.

KOR activation. As shown in Figure 1A, GABA binds to its postsynaptic site on a dopaminergic cell. This binding action opens Cl- channels which polarizes the cell and inhibits the release of dopamine at the synapse with the terminal neuron. Figure 1B depicts how the activation of presynaptic MOR

receptors on a GABAergic neuron triggers a G-protein sequence which inhibits the release of GABA. The resulting reduction of GABA at the dopaminergic neuron reverses the usual suppression of dopamine release, thus increasing the availability of dopamine at the terminal neuron. Morphine and MOR agonists applied directly to the VTA will establish the described increases in dopamine in the NAc in a dose-dependent manner (Latimer et al., 1987) and produce a dose-related conditioned place preference (Bals-Kubik et al., 1993; Spanagel et al., 1992). It is well established that increases in the synaptic availability of dopamine, in particular increased activation of dopamine-D1 receptors in the nucleus accumbens (NAc), mediates drug-induced conditioned place preference (Acquas et al., 1989; Spanagel et al., 1992; Shippenberg et al., 1993).

In essence, as dopamine flow increases in the NAc, it influences the conditioned reinforcing effects of contextual stimuli. If a dopamine antagonist, haloperidol, is injected into the NAc then morphine-induced place preference is attenuated (Shippenberg et al., 1993). 6-OHDA lesions of the NAc, the major termination site of A10 dopaminergic neurons from the VTA, will attenuate heroin-induced conditioned place preference (Spyraki et al., 1983). Similarly, if subjects are given a pretreatment of haloperidol prior to heroin injections then heroin-induced conditioned place preference is attenuated (Spyraki et al., 1983). The administration of haloperidol will also block place preference established by pairing a contextual stimulus with natural reinforcers, such as food (Spyraki et al., 1982).

Unlike MOR agonists, KOR agonists tend to establish conditioned place aversion when paired with contextual stimuli (Mucha and Herz, 1985; Shippenberg and Herz, 1991; Bals-Kubik et al., 1993; Shippenberg et al., 1993). These effects are shown to be related to a dose-dependent reduction in extracellular dopamine release after acute administration of KOR agonists (Gehrke and Chefer 2008). In general, KOR agonists reduce extracellular dopamine by inhibiting the release of dopamine from the VTA into the mesolimibic dopaminergic pathway (Bals-Kubik et al., 1993; Spanagel et al., 1992; Margolis et al., 2003; Leyton et al., 1992). Specifically, KOR activation partially

mediates dopamine release into the NAc (Ebner et al., 2010). As illustrated in Figure 1C, KOR agonists activate G-protein-coupled inwardly rectifying potassium (GIRK) channels (Margolis et al., 2003). GIRK channels are G-protein-gated ion channels that, when opened, allow the cell to become more permeable to potassium, the cell hyperpolarizes and inhibit dopamine release. Hence, the reduced flow of dopamine following KOR activation is a result of the direct inhibition of dopaminergic neurons. The KOR-induced dopamine inhibition in the VTA can be blocked by administering KOR antagonists (Margolis et al., 2003).

The general rule is that MORs disinhibit dopamine transmission and KORs inhibit dopamine transmission, as explained prior, but this relation is only true in certain neurons. Although MOR and KOR agonists typically act on either primary, secondary or tertiary dopaminergic neurons, Margolis et al. (2003) note that neurons in the VTA can not fall under this simple classification, as there are an extensive variety of receptor combinations and neurotransmitter possibilities. Margolis et al. (2003) reported finding neurons that were "...MOR agonist-inhibited, KOR agonist-inhibited, inhibited by both MOR and KOR agonists, and inhibited by neither" (P9984). In general, there are fewer KORs than MORs both in the NAc and the VTA (Mansour et al., 1988), but the diversity of neurons within the VTA produces a matrix of individual cell responses depending on the opioid receptors present on a particular cell. Ford et al. (2006) suggested receptor sensitivity of VTA neurons is related to projection sites. During KOR and MOR activation in the VTA, neurons fire differently depending on their termination site. Specifically, a majority of KOR-sensitive neurons project into the NAc whereas a majority of MOR-sensitive neurons project into the basolateral amygdala (Ford et al., 2006). Until more research exposes the organization of individual neurons of the dopaminergic pathway, investigating dopamine levels after drug administration within specific nuclei would be more efficient than at the cellular level. However, measurements of neurochemical changes within specific nuclei may also be inconclusive. There is an inconsistency within the literature concerning whether KOR activation in the VTA (Bals-Kubik et al., 1993; Margolis et al., 2003; Leyton et al., 1992)

or within the NAc (Spanagel et al., 1992; Shippenberg et al., 1993) is responsible for the overall reduction of dopamine in the NAc. The spontaneous action of the VTA could differ between awake and anesthetized animals and the different methods used in researching neurochemical changes in these brain regions may account for these discrepancies. The alternative approach is utilizing behavioral assays, which arguably is a more dependable method of predicting drug-related reward rather than measuring responses at the neuronal level.

Generally, the rewarding and aversive effects of MOR and KOR agonists can be blocked by pre-injections of MOR and KOR antagonists, respectfully (Acquas et al., 1989; Zhang et al., 2005; Braida et al., 2008). Moreover, the behavioral effects of other drugs of abuse may be reduced by the co-administration of kappa opioid receptor (KOR) agonists. For instance, the KOR agonist U-69593 prevents cocaine-induced enhancement of brain stimulation reward (Tomasiewicz et al., 2008) and the KOR agonist, U-50488, decreases cocaine-induced locomotor activity (Crawford et al., 1995). Of special interest is the finding that U-5048- attenuates morphine-induced conditioned place preference and locomotor activity (Huang et al., 2007).

Neuroanatomical Substrates of Conditioned Reward

Blockade of all dopamine transmission will attenuate rewarding as well as aversive properties of place conditioning (Acquas et al., 1989) suggesting the behavioral effect of conditioning contextual stimuli is mediated by the dopaminergic system. However, operant administration of natural rewards (i.e., food and water) in comparison to cocaine will produce different phasic firing of dopaminergic neurons in the NAc (Carelli et al., 2000). In addition, animals that self-administer a drug tend to have more dopamine release within the NAc compared to those with yoked drug administration (Di Cano et al., 1998a). A central finding of conditioned reward is that regardless of how a drug is paired with environmental stimuli, whether it is an active process via operant administration or a passive, like yoked administration or place conditioning, an increase in NAc dopamine release occurs. Di Caino et al.'s (1998a) study found dopamine levels for both self-

administration and yoked administration groups increase by the *same* amount when the animal was presented with a drug-paired, conditioned stimulus in a drug-free state. Further, post-conditioning exposure to conditioned stimuli, while in a drug-free state, will produce increases motor activity and a rise in extracellular dopamine levels in the NAc (Di Cano et al., 1998b).

SCIENTIFIC AIMS

The primary aim of the present study (experiment 1) was to determine if salvinorin A was capable of attenuating morphine-established CPP similar to synthetic KOR agonist U-50488's attenuation of morphine-induced CPP (Huang et al., 2007). This study also investigated the conditioning effects of salvinorin A when administered alone. At least one laboratory has reported that very low doses of salvinorin A are capable of establishing CPP (Braida et al., 2007; 2008). However, typical results show that salvinorin A as well as synthetic KOR agonists produce conditioned place aversion (Zhang et al., 2005; Shippenberg & Herz, 1987; Huang et al., 2007; Walker, 2009). Only two known studies (Braida et al., 2008; Walker, 2009) have assessed conditioning effects of salvinorin A in CPP using rats, with conflicting results. However, it was noted the two studies prepared salvinorin A in different vehicles. Braida et al. (2008) used an ethanol/TWEEN80/sterile water mixture (1:1:8), whereas Walker (2009) used 75% dimethylsulfoxide (DMSO), a polar aprotic solvent. Previous investigations in our laboratory have utilized DMSO as salvinorin A's vehicle due to difficulties maintaining salvinorin A in an ethanol/TWEEN80/water solution (Walker, 2009). Due to prior results of DMSO establishing conditioned place aversion, a secondary aim (experiment 2) was to investigate the influence of the vehicle used to dissolve salvinorin A on place conditioning. Therefore, experiment 2 systematically compared the conditioning effects of salvinorin A prepared in two reported vehicles, DMSO/water and ethanol/TWEEN80/water.

EXPERIMENT 1

Methods

Subjects

Subjects were 36 adult, male Sprague Dawley rats obtained from Charles River Laboratories (Portage, MI). Subjects had prior experience with handling and had exposure to operant conditioning, but were naive to conditioned place preference chambers, injections, and drug treatment. Subject ages ranged from 4 months to 12 months old and were counterbalanced among treatment groups. Animals were singly housed in polycarbonate cages with corn cob bedding and *ad libitum* access to food and water. Animal housing facilities were maintained on a 12/12-h, light/dark cycle with the lights on from 0700 to 1900 and constant temperature (20±2°C).

Animals used in these studies were housed, handled, treated, and cared for in a humane and ethical manner in accordance with NIH guidelines (*Guide for the laboratory care and use of animals, Eighth Edition*. Accessible here http://oacu.od.nih.gov/regs/index.htm) and the animal use protocol was reviewed and approved by the Western Michigan University Institutional Animal Care and Use Committee.

Apparatus

Eight custom-designed chambers constructed from clear acrylic and were fitted within a Versamax® animal monitoring system, equipped with infrared beams, which detected movement along an XY coordinate plane (AccuScan Instruments, Columbus, OH). Each chamber measured 40 cm long X 40 cm wide X 40 cm high, at 0.5 cm thick, and was divided into two equal compartments (40 X 20 cm) by an acrylic wall. The wall had a 10 X 10 cm opening to allow animals to pass between compartments during habituation and testing sessions. The opening was covered with clear acrylic during conditioning trials to restrict animal movement to one compartment. Each compartment contained distinct visual and tactile cues. One compartment was equipped with black and white horizontal lines on each wall and a smooth, aluminum floor with 1 cm diameter holes spaced 0.5 cm

apart. The adjacent compartment was equipped with black and white vertical lines on each wall and a textured plastic floor (Figure 2).

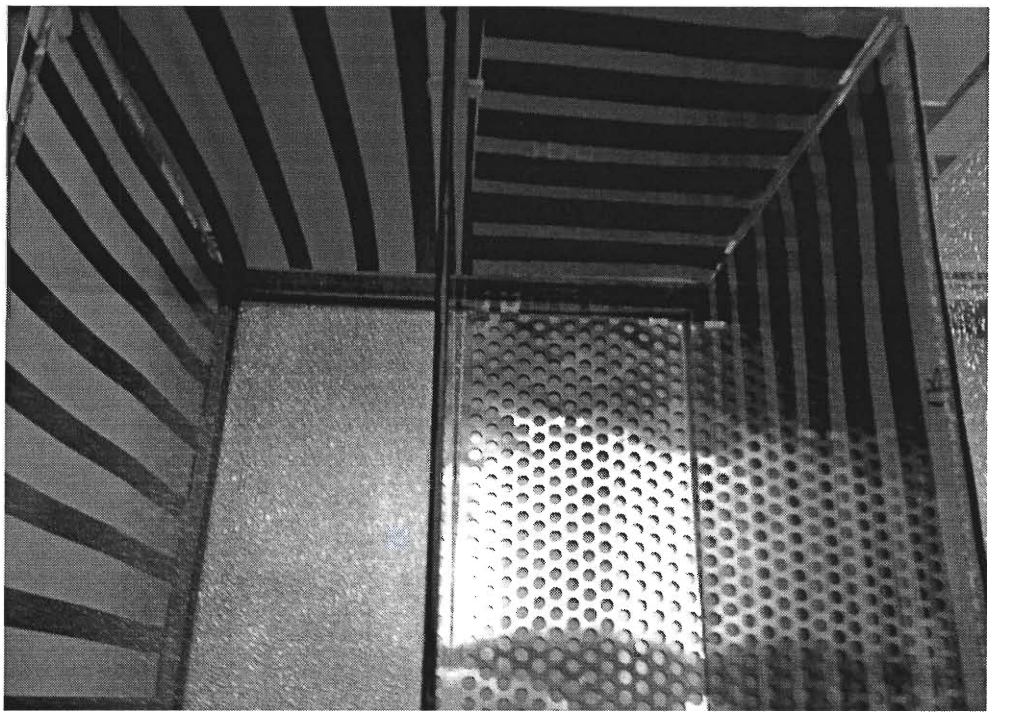

Figure 2. Place conditioning apparatus used in Experiment 1 and Experiment 2.

Drugs

Salvinorin A was synthesized by Dr. Thomas Munro and donated by Harvard McLean Hospital (Belmont, MA). Salvinorin A (0.4mg/kg) solution was prepared fresh daily by dissolving it in dimethysulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO) and then diluting it with sterile water to form a 75% DMSO solution. Morphine (10 mg/kg) was provided by the National Institute of Drug Abuse (NIDA; Bethesda, MD) and was dissolved in 0.9% saline. Drugs were administered via intraperitoneal (I.P.) injection using 1 cc Monoject syringes at a 1 ml/kg injection volume.

Habituation Trials

Animals were acclimated to the chambers for 15 minutes a day for three consecutive days. Animals were placed in the center of the chamber, in the center wall's opening, and were then

allowed free access to both sides of the chamber for 15 minutes. Horizontal activity and time spent in each compartment was recorded for each rat. On the last habituation trial, time spent in each compartment was used to assign drug-compartment and vehicle-compartment during subsequent conditioning trials. The compartment in which the animals spent the least amount of time was designated as that individual's drug-paired side. For one squad (consisting of two animals per treatment group), the drug side was inadvertently assigned to their preferred environment on habituation day. This did not appear to influence the outcome of the study so the data for these animals was not excluded from analysis.

Place Conditioning Trials

Rats were randomly assigned to one of the following four treatment groups: 10 mg/kg morphine vs. saline (MOR, n = 9), 0.4 mg/kg salvinorin A vs. DMSO (SA, n = 9), 0.4 mg/kg salvinorin A + 10 mg/kg morphine vs. DMSO + saline (MOR + SA, n = 9), and DMSO vs. saline (DMSO, n = 9). The MOR group and SA groups served as a control for the MOR + SA group. Due to inconsistent reports in the literature concerning the conditioning effect of salvinorin A, the DMSO group functioned as another control group to assess the conditioning effect of salvinorin A. Conditioning trials were conducted in squads with a maximum of eight animals per squad, drug treatments were counterbalanced among squads and chambers. All animals were injected within 5 minutes before being placed in their assigned chamber for 30 min. Drug and vehicle conditions alternated daily. Animals were given drug the first conditioning day, vehicle the second day and so forth for eight consecutive days. Chambers were cleaned with lemon-scented cleaning wipes before and after each squad.

CPP Test

The day following the last conditioning trial was designated as test day. The procedure was the same as habituation day. Animals were allowed free access to either environment in the CPP chamber as locomotor activity and time spent in each compartment was recorded.

Statistical Analysis

All data were analyzed with IBM SPSS Statistics 20 (SPSS Inc., Chicago IL). Locomotor activity was recorded as horizontal infrared beam breaks within the chamber during conditioning trials. A two factor repeated measures ANOVA was conducted on locomotor activity, with injection type as a between subjects factor and conditioning trial a within subjects factor. Bonferroni post hoc tests were used to determine any significant differences between particular drug treatment groups. In addition, the average activity across all four drug conditioning trials was calculated for each group. A one factor ANOVA and Bonferroni post-tests were conducted on these data to determine any significant effect of drug treatment on activity. To assess the effects of conditioning on behavior during the test trial, difference scores were determined for each animal by calculating time spent in drug-compartment minus time spent in the vehicle-compartment. This measure is the most common dependent variable for analyzing CPP (Bardo and Bevins, 2000). A one factor ANOVA followed by Bonferroni post-tests were conducted on the difference scores.

Results

Locomotor Activity

Figure 3 depicts the locomotor activity recorded during the eight conditioning trials. Regardless of DMSO or saline, the highest locomotor activity was seen on the first conditioning day followed by a steady decline in activity with repeated trials. In contrast, morphine-induced locomotor activity steadily increased with repeated dosing. Salvinorin A appeared to suppress locomotor activity relative to vehicle. Salvinorin A + morphine produced the least amount of activity compared to other treatment groups. A repeated measures ANOVA (treatment group, conditioning trial) shows there was a significant main effect of conditioning day [F (7, 224) = 6.56, $p < .0001$], drug treatment [F (3,32) = 3.51, $p < .03$]and a significant interaction between the drug treatment and conditioning day [F (21, 224) = 6.23, $p < .0001$]. An additional one factor ANOVA was run on the group average per drug conditioning trial. Results of the one factor ANOVA found significant effect

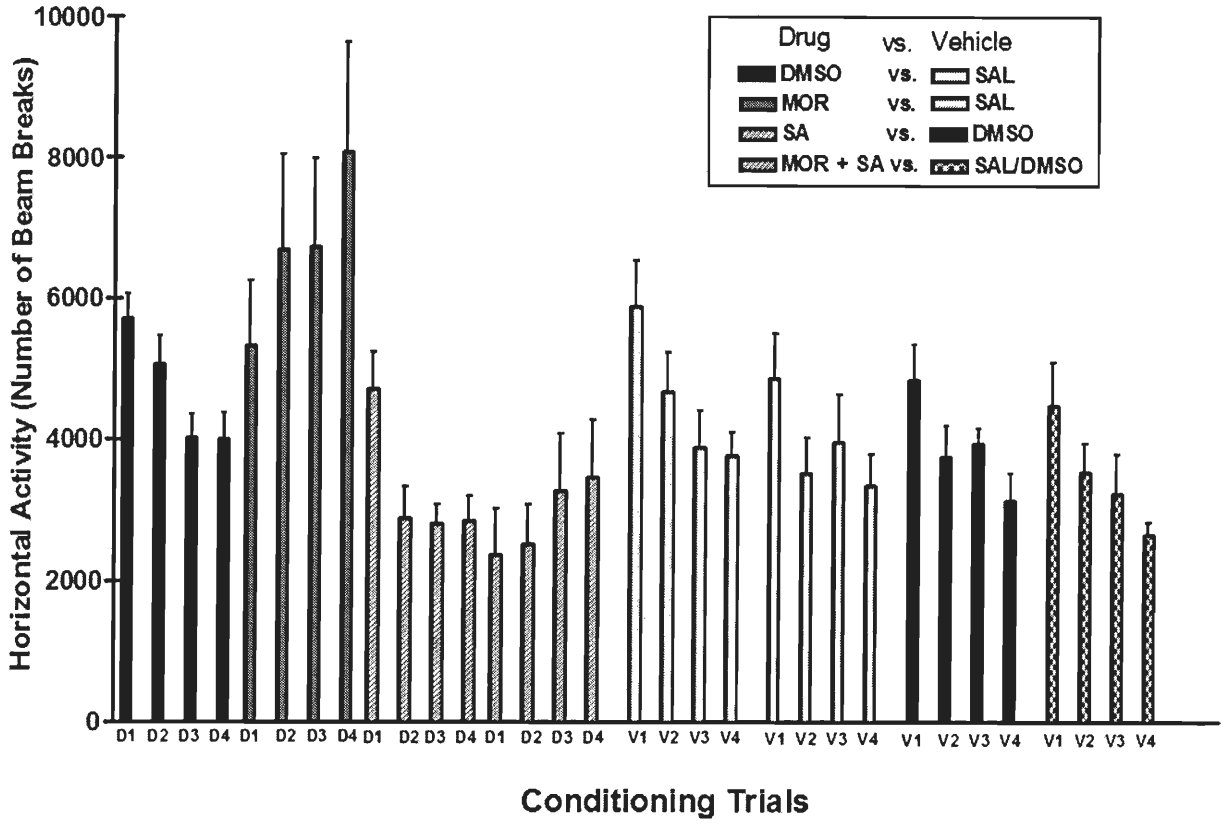

Figure 3. Mean (n = 9) locomotor activity per treatment group during drug and vehicle conditioning in Experiment 1. Error bars represent standard error.

of drug treatment [F (3, 32) = 5.43, p < .01] and Bonferroni post-tests showed activity of the MOR group was significantly different from that of the SA group (p<0.05) and the MOR+SA group (p<0.01). This difference indicates that the co-administration of salvinorin A with morphine attenuated the typical locomotor activity seen with morphine alone.

When excluding vehicle conditioning data from the analysis, repeated measures ANOVA (treatment group, conditioning trial) reveals a significant main effect of drug treatment [F (3, 32) = 5.44, p < .01] and a significant interaction between the conditioning day and drug treatment [F (9, 96) = 9.86, p <.001]. A Bonferroni post-hoc analysis shows a significant difference between MOR and MOR + SA (p = .006) and between MOR and SA (p = .016).

Conditioned Place Preference

Conditioned place preference was calculated for individual animals by subtracting the time spent on the vehicle-paired chamber from the time spent on the drug-paired chamber during the CPP test. Group means (± S.E.M.) of difference scores are plotted in Figure 4. Bars in a positive

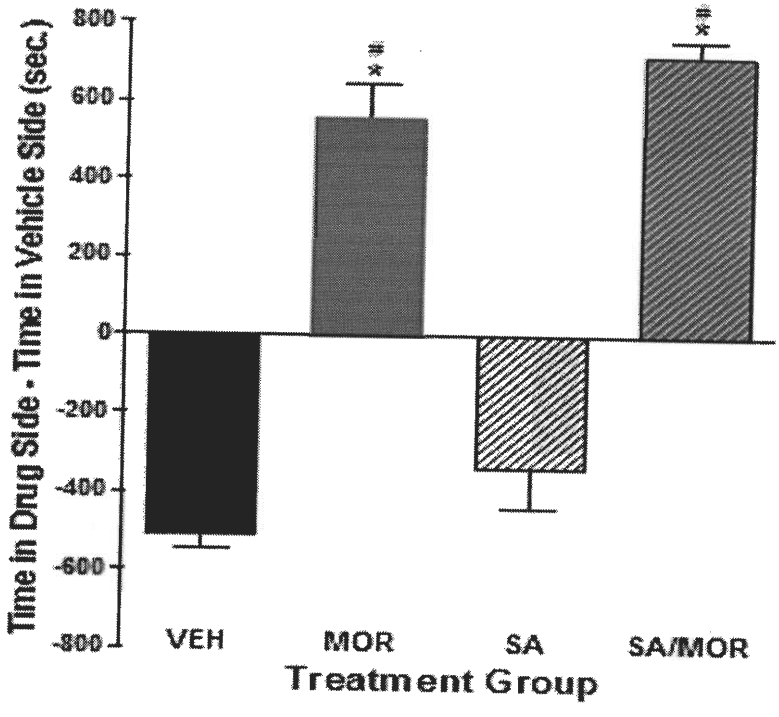

Figure 4. Experiment 1 conditioned place preference for the drug-paired chamber on test day. Error bars represent standard error. # Represents statistically significant from VEH group and * represents statistically significant from SA group.

direction indicate place preference (i.e., more time spent in drug-paired chamber), whereas bars in a negative direction indicate place aversion (i.e., less time spent in the drug-paired chamber). Results of a one factor ANOVA found a significant effect of drug treatment on chamber preference during test day [$F(3, 32) = 71.67$, $p < 0.001$]. Results were further examined using Bonferroni post-hoc tests. As depicted in Figure 4, significant differences were found between MOR + SA and DMSO ($p<.001$), MOR + SA and SA ($p<.001$), MOR and DMSO ($p<.001$), and MOR and SA ($p<.001$). That is, subjects in the DMSO and SA groups tended to spend less time in their designated drug compartment compared

to subjects in the MOR and MOR + SA groups who displayed CPP for the drug-paired environment. There was no statistically significance between the SA and DMSO groups or between the MOR + SA and MOR groups. However, there was a slight increase in time spent on the drug side for rats in the MOR + SA group (M=719.64, SD =117.94) compared to those in the MOR group (M = 553.73, SD = 281.23).

EXPERIMENT 2

Introduction

Results of Experiment 1 indicated that salvinorin A produces conditioned place aversion. These results are consistent with those of Zhang et al. (2005) but are inconsistent with the findings of Braida et al. (2008), who reported that low doses (0.001 mg/kg – 0.04 mg/kg) of salvinorin A produces conditioned place preference. Prior studies investigating place conditioning of salvinorin A utilized an ethanol/TWEEN80/water (1:1:8) mixture to dissolve SA (Zhang et al., 2005, Braida et al., 2008). However, results of Experiment 1 of the present study utilized DMSO vehicle due to difficulties in our laboratory maintaining salvinorin A in an ethanol/TWEEN80/water solution. These difficulties were confirmed by a previous report of uneven distributions of SA when prepared in TWEEN80 (Valdés III, 1994). Walker (2009) also used a 75% DMSO solution in attempts to replicate Braida's et al. (2008) study. Walker (2009) investigated a low dose of salvinorin (0.04 mg/kg; S. C.) within the range that Braida et al. (2008) reported established CPP as well as at a ten-fold higher dose. Contradictory to Braida et al. (2008), Walker (2009) found both doses of salvinorin A established conditioned place aversion. Experiment 1 in the present study found 0.4 mg/kg produced modest conditioned place aversion, supporting Walker's (2009) results. In addition, Experiment 1 revealed that DMSO alone established conditioned place aversion relative to saline, making it difficult to determine whether salvinorin A prepared in this manner induced place aversion due to the aversive properties of DMSO or due to the effects of salvinorin A. Therefore, to assess whether the results of Braida et al. (2008) and Walker (2009) were due to the different vehicles

employed, Experiment 2 systematically compared the place conditioning of salvinorin A dissolved in DMSO versus ethanol/TWEEN80 /water.

Methods

Subjects and Apparatus

Subjects were 32 experimentally naïve, adult, male Sprague Dawley rats obtained from Charles River Laboratories (Portage, MI). Housing and animal care were described in Experiment 1. The apparatus used in Experiment 2 was the same apparatus described in Experiment 1.

Drugs

Preparation of salvinorin A in DMSO was the same as in Experiment 1. For comparison, salvinorin A (0.4mg/kg) was also prepared daily in ethanol, TWEEN80, and sterile water (1:1:8 proportion by volume).

Habituation, Conditioning and Test Trials

Animals were randomly assigned to one of five treatment groups: DMSO vehicle vs. saline (DMSO, n =6), ethanol/TWEEN80/water vehicle vs. saline (TWN80, n = 6), Salvinorin A in DMSO vs. DMSO vehicle (0.4mg/kg; SA + DMSO, n = 7), Salvinorin A in ethanol/TWEEN80/water vs. ethanol/TWEEN80/water vehicle (0.4mg/kg; SA + TWN80, n = 7) and finally a saline vs. saline control group (SAL, n = 6). The habituation, conditioning and testing procedures were the same as in experiment 1.

Statistical Analysis

All data were analyzed with IBM SPSS Statistics 20 (SPSS Inc., Chicago IL). Locomotor activity during conditioning trials and difference scores obtained from test trial were analyzed the same as in experiment 1.

Results

Locomotor Activity

All treatment groups exhibited the most locomotor activity on the first conditioning day, with a steady decrease in locomotor activity across conditioning trials (Figure 5). This trend was seen

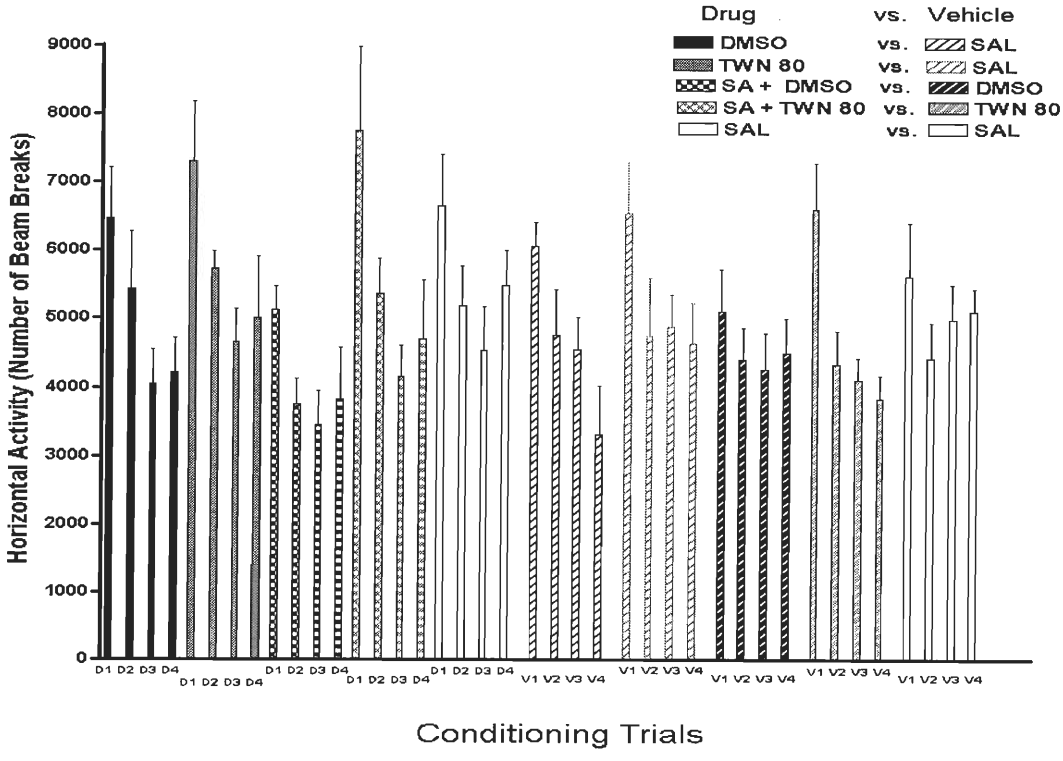

Figure 5. Experiment 2 mean (n = 5, 6) locomotor activity per treatment group during conditioning trials. Error bars represent standard error.

both for drug conditioning days and for vehicle conditioning days. A repeated measures ANOVA (treatment group, conditioning trial) revealed a significant main effect of conditioning day [F (7, 133) = 15.71, $p < .001$] but no significant effect of treatment group or interaction between group and trial. Bonferroni post-tests were not significant.

Conditioned Place Preference

As seen in figure 6, difference scores were calculated and plotted similar to Experiment 1.

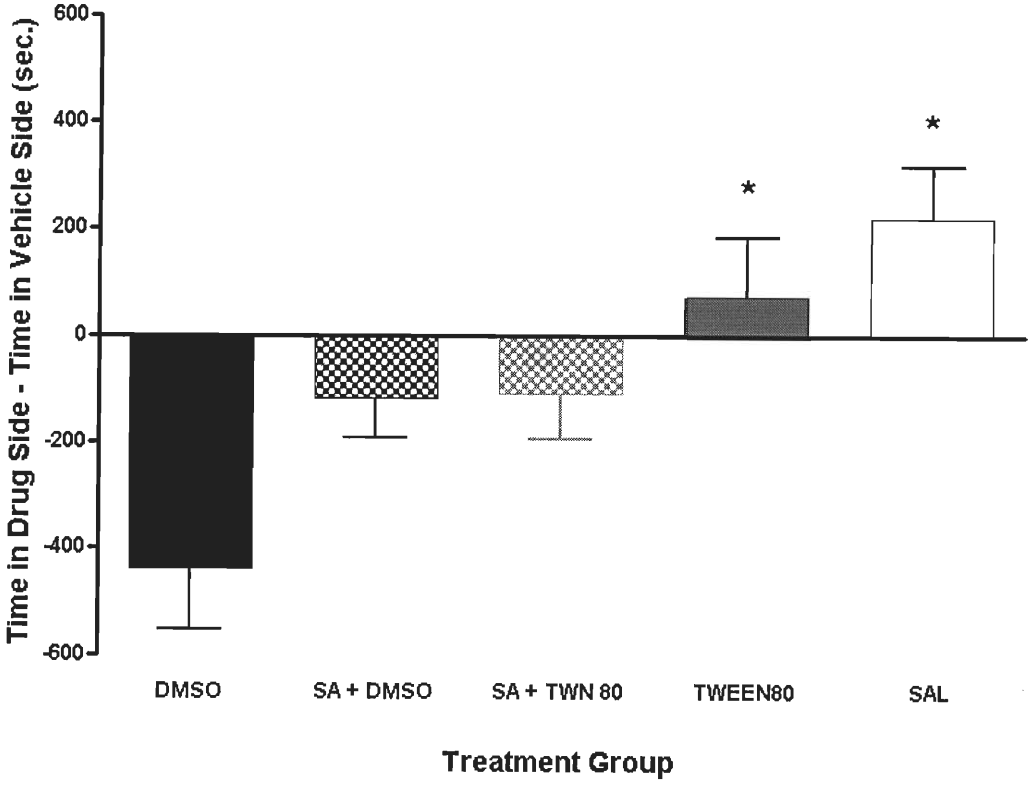

Figure 6. Experiment 2 conditioned place preference for the drug-paired environment. Error bars represent standard error. * Represents statistically significant from DMSO.

The DMSO vs. saline treatment group spent more time in the saline-paired compartment, indicating a conditioned aversion to the compartment paired with DMSO injections. All other treatment groups showed no evidence of conditioned place preference or aversion. Results of a one factor ANOVA showed a significant main effect of drug treatment [$F(4, 27) = 6.16$, $p < 0.01$]. Bonferroni post-hoc tests showed a significant difference between SAL and DMSO ($p < 0.001$) and between DMSO and TWN80 ($p < .05$). Regardless of the vehicle used to dissolve salvinorin A, resulting place conditioning was almost identical: the mean difference score for the SA + DMSO group was -117.51 sec (SD = 202.45) and it was -110.80 sec (SD = 225.88) for the SA + TWN80 group. Neither group was significantly different from the saline-treated group (M = 219.07, SD = 238.97).

DISCUSSION

Results of Experiment 1 found that 0.4 mg/kg salvinorin A produced conditioned place aversion. These findings are consistent with those of Zhang et al. (2005), who reported salvinorin A produced conditioned place aversion in mice, and are consistent with those of Braida et al. (2008), who reported salvinorin A at higher doses produced conditioned place aversion in rats. However, Braida et al. (2008) also reported that extremely low doses of salvinorin A established conditioned place preference. Walker (2009) attempted to replicate Braida's et al. (2008) study and found that salvinorin A produced conditioned place aversion, even at the low doses Braida et al. (2008) reported to produce conditioned place preference. It is possible that the discrepancy between Walker's (2009) and Braida's et al. (2008) results is due to the vehicle used to dissolve salvinorin A. This supposition is supported somewhat by current findings that DMSO seemed to establish a conditioned place aversion as other published studies of CPP using DMSO have not explored DMSO compared to a saline control. To investigate this possibility further, experiment 2 systematically compared the place conditioning effects of salvinorin A (0.4 mg/kg) dissolved in two different vehicles. Experiment 2 found salvinorin A dissolved in 75% DMSO induced conditioned aversion almost identical to salvinorin A dissolved in an ethanol/TWEEN80/water vehicle. Although it is important to note that salvinorin A may have slightly attenuated DMSO-conditioned aversion, whether this result was related analgesic effects or potential rewarding effects KOR activation is unknown.

Therefore, the results from Experiment 2 suggest that the discrepancies between Braida et al. (2008) and Walker (2009) were independent of the vehicle used. However, it should be noted that Braida et al. (2008) and Walker (2009) both administered drug via subcutaneous injection whereas the current study administered drug via intraperitoneal injection (I.P). Another difference was Braida's et al. (2008) use of Wistar rats, whereas Walker (2009) and the present study used Sprague-Dawley rats. Zhang et al. (2005) employed a dose of 1.0 mg/kg and 3.2 mg/kg salvinorin A

in the aforementioned ethanol/TWEEN80/water mix (I.P.) using mice and reported conditioned place aversion. Rat strain and route of administration should be explored in future research.

The lack of consistent findings in the pre-clinical examinations of salvinorin A's abuse liability raises questions concerning the recreational use of salvinorin A in humans. Typically, other hallucinogenic drugs (e.g., LSD) are not self-administrated in nonhumans, nor do they produce conditioned place preference in nonhumans. Thus, CPP may not be an adequate assay of human abuse potential for hallucinogens. Like other hallucinogens, human use of salvinorin A may be related to its heavy influence on priming (Weil, 1998; Valdés III, 1994) and changes in sensitivity to other sensory cues like light and sound (Valdés III et al., 1983). Curanderos would instruct the patient on how to navigate their visions, explaining what they will experience, and send them to a dark and quiet place to encounter spiritual visitations (Valdés III et al., 1983; Valdés III, 1994). Valdés (1994) notes that, "The curandero spent hours before each session describing what we would see. This had a tremendous influence on my second experience" (p. 9). Valdés (1994) also reported that during his investigation of Mazatec rituals, the more intense and longer lasting visions occurred when the setting was dark and quiet. In a personal account, Valdés (1994) noted that when the Mexican village was noisy, the visions were infrequent and concentration was needed to "bring back" images. However, once he settled that night to sleep in a dark and quiet room, the visions returned at full strength. These observations bring about questions concerning the role of other sensory perceptions in understanding the sensitivity of salvinorin A's effects. Unfortunately, the human subjective drug experience is difficult to replicate in animal models.

Locomotor activity in addition to condition place preference testing is also commonly used in preclinical screening of CNS active drugs and assists in determine the pharmacological actions of novel compounds. The current study is the first known study to demonstrate that salvinorin A significantly attenuates morphine-induced locomotor activity. These findings are consistent with reports that a synthetic KOR agonist, U-50488, decreases morphine-induced locomotor activity

(Huang et al., 2007) as well as cocaine-induced locomotor activity (Crawford et al., 1995). Reduction in morphine-induced locomotor activity by a KOR agonist is likely related to the ability of KOR agonists to reduce dopamine levels in the NAc. Zhang et al. (2005) reported both decreases in morphine-induced locomotor activity and decreases in extracellular dopamine after administration of salvinorin A. Typically, drugs that increase NAc dopamine also increase locomotor activity. Locomotor activity can be stimulated by direct increases in dopamine into the NAc. This effect is blocked by dopamine antagonists injected into the NAc and amplified by amphetamines (Pijnenburg et al., 1975). Although both locomotor activity and place conditioning have been previously shown to be mediated by drug-induced increases in dopamine levels, the present study found that 0.4 mg/kg salvinorin A attenuates MOR-induced locomotor activity, but failed to inhibit MOR mediated place conditioning, suggesting these behaviors can be pharmacologically altered independent of one another. However, it is possible that higher doses of salvinorin A are required to block MOR-induced CPP.

Ebner (2010) reported that 0.25 mg/kg salvinorin A did not alter extracellular levels of dopamine in the NAc, but 2.0 mg/kg significantly suppressed dopamine release into the NAc compared to vehicle. Moreover, Zhang et al. (2005) showed that higher doses of salvinorin A that produced place aversion (1.0 mg/ kg and 3.2mg/kg; I.P.) were accompanied by significant suppression of dopamine release. Furthermore, at least two other studies have found synthetic KOR agonists attenuate morphine-induced CPP. For example, Huang et al. (2007) co-administered U-50488 (8 mg/kg; I.P.) with morphine (10 mg/kg; I.P.) and eliminated morphine-induced conditioned preference and established conditioned place aversion. In a similar study, Bolanos et al. (1996) found a pretreatment of U -50488 (2–10 mg/kg; S. C.) prior to injections of morphine (0.1 mg/kg to 8 mg/kg; I.P.) blocked morphine-induced CPP in rats 10-17 days old, but not for rats 35 days old. Provided synthetic KOR agonists and salvinorin A are comparable in potency and efficacy, a stronger dose of salvinorin A could yield results comparable to the U-50488 seen in Huang et al. (2007) and

Bolanos et al. (1996). In an investigations employing a drug discrimination task to compare salvinorin A to synthetic KOR agonists, Baker et al. (2009) reported similar potencies between U-50488 and salvinorin A. Although equivalent in behavioral potency, the efficacy at opening GIRK channels differs between synthetic KOR agonists and salvinorin A. When comparing the potassium conductance through GIRK channels, Chavkin et al. (2004) found salvinorin A to be more efficacious than its synthetic rival, U-50488. Because more efficacious drugs tend to be more selective, salvinorin A could interact differently at opioid receptors than synthetic KOR agonists.

Future research should focus on the direct comparisons between synthetic KOR agonists and salvinorin A, especially if they are to be considered for use in therapeutic treatments. If these compounds act similarly, then it can be assumed higher doses salvinorin A will suppress dopamine release into the NAc and may be considered for use in addiction research. If synthetic vs. natural compounds act differently on neurons projecting to other regions of the brain, such as the NAc, knowing the disparities between salvinorin A and its synthetic derivatives is crucial in determining the proper compound to select for specific therapies. Direct comparisons between synthetic and natural kappa opioid agonists must be studied prior to classifying them as interchangeable.

In conclusion, the present study demonstrated that salvinorin A (0.4 mg/kg) attenuated morphine-induced locomotor activity during CPP, but this dose of salvinorin A was not able to mitigate morphine-induced conditioned place preference. These results highlight KOR and MOR interaction on only one of two distinct behavioral effects, implying that different factors are involved in reward processing and drug-stimulated locomotor activity. In addition, experiment 2 found that salvinorin A establishes modest conditioned place aversion and the vehicle DMSO established significant conditioned place aversion, but neither DMSO nor ethanol/TWEEN80/water influenced the place conditioning of salvinorin A.

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APPENDIX

WESTERN MICHIGAN UNIVERSITY

Centennial 1903-2003 Celebration

Institutional Animal Care and Use Committee

Date: March 10, 2010

To: Lisa Baker, Principal Investigator

From: Robert Eversole, Chair

Re: IACUC Protocol No. 10-02-05

Your protocol titled "Conditioned Place Preference Procedures in Rats" 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: March 10, 2011

Walwood Hall, Kalamazoo, MI 49008-5456
PHONE: (269) 387-8293 FAX: (269) 387-8276