Dose-related Behavioral, Subjective, Endocrine and Psychophysiological Effects of the Kappa Opioid Agonist Salvinorin A in Humans

Supplemental Information

Recruitment

Subjects were recruited by print and electronic advertisements placed in the community and by word of mouth. After a brief telephone contact, interested and potentially eligible subjects were invited in for a rigorous face-to-face screening.

Screening Process

Written informed consent was obtained during which subjects were informed about the potential for adverse effects of Salvinorin A (SA) including dysphoria, psychosis and anxiety. After obtaining consent, subjects (18-55 years) underwent a structured psychiatric interview for DSM-IV-TR (1) and were carefully screened for participation by a research assistant and research psychiatrist. In order to enhance subject safety, given the limited knowledge on the effects of SA in humans, subjects were included only if they were psychiatrically and medically healthy adults who had been previously exposed to Salvia or SA. Since Salvia users characteristically use other drugs such as cannabis and hallucinogens (2), subjects with exposure to other drugs were included in order that the sample be representative. Subjects were excluded if they were ever diagnosed with an Axis I psychotic or mood disorder, had current substance dependence (except nicotine), major or unstable medical problems, were pregnant or breastfeeding, or had a family history of psychosis in a first-degree relative. With their consent, the history provided by subjects was confirmed by a telephone interview conducted with a spouse or family member identified by the subject during screening. A general physical and neurological examination, electrocardiogram and laboratory tests (serum electrolytes, liver function tests, complete blood count with differential and urine toxicology)

were also conducted. Psychosis proneness was measured using the Schizotypal Personality Questionnaire. Subjects were familiarized with the vaporizer used for delivering SA. Subjects were instructed to refrain from alcohol, caffeine, and illicit drugs or prescription drugs not approved by the research team for a week before the first test day and throughout study participation.

Table S1. Schedule of Procedures

Time (minutes)	Procedure
−60	Confirmation of abstinence from caffeine, alcohol, drugs and unapproved medications Urine drug screen and pregnancy test Vital signs and placement of an IV line and pulse oximeter
−30	Behavioral & subjective assessments: VAS, PANSS, PSI, CADSS, HRS. Blood sampling for hormonal assays and SA and SB levels Vital signs
0	Active SA or Placebo by inhalation Vital signs every 5 minutes until +30 Blood sampling every 5 minutes from +10 until +30 Resting state EEG for 3 minutes from +3 till +6
+7	Cognitive testing: Digit forward, Digit Backward, Letter Number Sequencing Test
+30	Behavioral & subjective assessments (rating for both peak effects after drug administration and current ratings): VAS, PANSS, PSI, CADSS, HRS. Blood sampling for hormonal assays and SA and SB levels Vital signs
+60	Vital signs
+90	Blood sampling for hormonal assays and SA and SB levels Vital signs PSI (rating for both peak effects after drug administration)
End of each day (+90)	Vital Signs Drug Liking Questionnaire, MMSE Field sobriety test and discharge by physician
Day after test days 1 and 3, and 1 and 3 months after study completion	Assessment for emergence of new psychiatric or medical problems Assessment of alterations in Salvia use

VAS, Visual Analog Scale; PANSS, Positive and Negative Syndrome Scale; PSI, Psychotomimetic States Inventory; CADSS, Clinician Administered Dissociative Symptoms Scale; HRS, Hallucinogen Rating Scale; SA, Salvinorin A; SB, Salvinorin B; EEG, electroencephalography; IV, intravenous; MMSE, Mini Mental State Examination.

Table S2. Subject Demographics

	Mean (SD)
Age (years)	23.8 (3.2)
Gender	9 Males, 1 Female
Education (years)	15.3 (1.2)
Schizotypy scores (SPQ)	2.8 (2.8)
Wisconsin Psychosis Proneness Scores	19.4 (12.1)
Intelligence Quotient (NART)	117.2 (7.1)
Smoking (cigarettes/month)	34.5 (79.3)
Family History (First Degree Relatives)	Substance Use Disorder: 1 (Alcohol Dependence) Mood disorder: 1 (Major Depressive Disorder) Psychotic Disorder: 0

SPQ, Schizotypal Personality Questionnaire; NART, National Adult Reading Test.

Table S3. Lifetime exposure to Substances

Substance	Number of subjects
Salvia	10
Marijuana	10
Cocaine	4
Mushrooms	4
Psilocybin	4
LSD	3
MDMA	3
DXM	2
Ecstasy	1
Synthetic Cannabinoids	1
Opium	1
Peyote	1

LSD, lysergic acid diethylamide; MDMA, 3,4-methylenedioxy-N-methylamphetamine; DXM, dextromethorphan.

Delivery of SA

SA was delivered via the "VP 600 Digital Herb Vaporizer" manufactured by Noble Vapor using a "paced inhalation procedure". The VP-600 heats up rapidly and has a built in fan to facilitate airflow into the vaporizer. This makes inhaling smoother and the fan also helps to control temperature by circulating air into the vaporizer. SA or placebo was placed in the bulb of the vaporizer. Once the target temperature (250-300°C) was reached the subject was instructed to inhale deeply and hold his or her breath for 30 seconds. This was repeated two additional times.

Behavioral and Subjective Measures

Visual Analog Scale of Feeling States (VAS)

Feeling states such as "high", "calm and relaxed", "anxious", "drowsy", "irritable", and "nervous" were measured using a self-reported visual analog scale (VAS). Subjects were instructed to indicate on a 0-100 mm line the perceived intensity of the feeling state experienced. Many of these subjective measures have been utilized in several previous studies at our center, where they showed high sensitivity to drug effects and convergent validity with other measures of these mood states and symptoms.

Psychotomimetic Symptoms

Psychotomimetic effects were measured using the Positive and Negative Syndrome Scale (PANSS) (3) and the Psychotomimetic States Inventory (PSI) (4). The PANSS contains subscales assessing symptom and behavior clusters, including positive symptoms (conceptual disorganization, hallucinatory behavior, suspiciousness, excitement, grandiosity and unusual thought content), negative symptoms (blunted affect, lack of spontaneity, poor rapport, emotional withdrawal, and motor retardation), and general symptoms (anxiety, depression, guilt feelings, somatic concern and disorientation), that are administered by a research assistant and scored from 1 (lowest) to 7 (highest). A modified version of the PANSS adapted for repeated

measurements within a short time-period was used. The same research coordinator rated all test days for each subject. Inter-rater reliability sessions were conducted every 1-2 months over the time period (~2 years) that this study was conducted and, for example, intraclass correlation coefficients for the PANSS were consistently greater than 0.85.

The Psychotomimetic State Inventory (PSI) is a measure of drug induced psychotomimetic states that has been used to capture the acute effects of cannabis and ketamine in a laboratory setting and retrospective data from cannabis users. The PSI facilitates the characterization of a wide range of dissociative/hallucinatory phenomena as well as cognitive disorganization associated with the administration of SA.

Perceptual Alterations

Perceptual alterations were measured using the Clinician Administered Dissociative Symptoms Scale (CADSS) (5) and the Hallucinogen Rating Scale (HRS). The CADSS has self and interviewer-administered items, and consists of 5 subscales evaluating aspects of altered environmental perception, time perception, body perception, feelings of unreality, and memory impairment. The CADSS has been used in other studies at this center with ketamine and delta-9-tetrahydrocannabinol (THC) (6, 7) and has been shown to be sensitive to the perceptual altering effects of drugs.

The Hallucinogen Rating Scale (HRS) is a 100 item scale that was developed to capture the subjective perceptual effects associated with the potent psychedelic drug N,N-dimethyl-tryptamine (DMT) (8). It has been used successfully to capture the effects of a number of psychedelic substances including LSD, psilocybin, etc. The HRS was also used in two prior studies with SA and was shown to be sensitive to SA effects (9, 10). The HRS was administered along with the CADSS to capture a wider range of perceptual alterations.

Cognitive Measures

Digit Forward

Subjects were read a series of digits at regular intervals by the rater. They were required to repeat the digits. The task progressively increases in load and provides an assessment of attention and immediate recall.

Digit Backward

Subjects were read a series of digits at regular intervals by the rater. They were required to repeat the digits in the reverse order. The task places a greater load on memory and provides a measure of learning and recall.

Letter Number Sequencing Test

The Letter-Number Sequencing Test provides an assessment of working memory. The tester verbally presents increasingly longer sequences of intermixed numbers and letters at a rate of 1/second. After each sequence, the participant is asked to repeat the numbers in ascending order first and then the letters in alphabetical order.

Neuroendocrine Assays

Blood was sampled before and at various time points after SA inhalation for plasma cortisol and prolactin levels. Blood was collected and immediately placed on ice, then centrifuged and the plasma aliquotted into appropriate vials and stored at -70°C. Plasma cortisol was measured by a Coat-A-Count procedure (radioimmunoassay) using a kit from Siemens. Plasma prolactin was measured using an ELISA (enzyme-linked immunosorbent assay) kit purchased from ALPCO.

Salvinorin A and Salvinorin B (SB) analysis

Blood was sampled before and at various time points after inhalation for SA and SB levels. Blood was collected and immediately placed on ice, then centrifuged and the plasma aliquotted into appropriate vials and stored at -70°C. SA and SB levels were measured by Dr. E. Thomas Everhart at the Drug Dependence Research Center, Langley Porter Psychiatric Institute, University of California as described elsewhere (11).

Method for Determination of Salvinorins A and B in DMSO/PEG-400 by APCI Liquid Chromatography / Mass Spectrometry (LC/MS/MS)

Sample Preparation

Two solutions of SA were prepared at a concentration of 4.00 mg/mL in DMSO–PEG-400 (25:75, v/v). One was prepared and allowed to sit on the laboratory benchtop at room temperature for 6 days, while the other was prepared just before sample analysis. Just before analysis, 100 µL of each solution was dissolved in 9.90 mL of ethyl acetate to provide two 10-mL solutions at concentrations of 40 µg/mL.

Calibration standards were prepared by spiking mixtures of SA and SB in the following amounts in 16 × 100 mm silanized screw-top culture tubes: 0, 10, 50, 100, 250, 500, 750, 1,000, and 2,000 ng/mL. Quality control (QC) samples were prepared in duplicate at the following concentrations: 50, 250, and 1,000 ng/mL. Each tube also contained a mixture of the internal standards, salvinorin A 4-CO_2CD_3 and salvinorin B 4-CO_2CD_3, in the amount of 2,000 ng each.

Twenty-five microliters (1,000 µg) of the ethyl acetate dilutions of the DMSO–PEG400 solutions were transferred to 16 × 100 mm silanized screw-top culture tubes, which had been supplemented with 2,000 ng of each of the internal standards. These samples were prepared in duplicate.

Extraction and Derivatization

All glass tubes were silanized. Each tube contained a mixture of the internal standards, salvinorin A 4-CO_2CD_3 and salvinorin B 4-CO_2CD_3, in the amount of 105 ng each. Since the internal standards were prepared in ethyl acetate, each tube was evaporated to dryness in the centrifugal evaporator. To each tube was added 1 mL of sample and 4 mL of an ethyl acetate-heptane (1:3, v/v) mixture. The tubes were vortex-mixed for 5 min and centrifuged at 3,000 rpm for 5 min. The aqueous layer was frozen in a dry ice–acetone bath, the organic layer decanted into a clean, silanized tube, and the solvent evaporated in the centrifugal evaporator. To each tube was added 2 mL of dichloromethane followed by evaporation again to remove residual water by azeotropic evaporation. To each tube was added 100 µL of a 5% (v/v) solution of acetic anhydride-d6 in methylene chloride (CH_2Cl_2) and 100 µL of 0.5% (w/v) N,N-dimethylaminopyridine in CH_2Cl_2. The tubes were capped and vortex-mixed for 0.5 h, and the reaction mixtures quenched by the addition of 100 µL of methanol and vortex mixing for another 5 min.

After evaporation of the solvent, the residue was partitioned between 4 mL of an ethyl acetate-heptane (4:1, v/v) mixture and 0.5 mL of 0.25 M sulfuric acid. Vortex-mixing for 5 min was followed by centrifuging for 5 min, freezing of the aqueous layer, and decanting of the organic layer into a clean tube with a wash with 0.5 mL of 5% aqueous $NaHCO_3$.

Vortex-mixing and centrifuging was followed by freezing of the aqueous layer, and decanting of the organic layer into a clean tube and evaporation to dryness in the centrifugal evaporator. The residues were reconstituted in 100 µL of MeOH, filtered through 0.2 µm filters, and transferred to 250 µL autosampler vial inserts. The inserts were placed in vials, capped, and loaded onto the autosampler tray.

Liquid Chromatography

LC analyses were performed on a Thermo Separation Products P4000 gradient pump. Separations were achieved on a Phenomenex Synergi Polar (4.6 × 150 mm, 4 µm particle size)

reversed-phase high-performance LC column employing 0.1% formic acid and methanol as the mobile phase components. All solvents were degassed before use, employing a Thermo Separation Products SCM1000 vacuum degasser equipped with the optional helium sparging module. The column was protected by the use of two Phenomenex Security Guard cartridges installed in tandem with the analytical column. The flow rate was 1 mL/min. Analyses were carried out with a linear gradient of 0.1% formic acid-methanol (50:50, v/v) to 100% methanol over a period of 15 min.

Mass Spectrometry

Mass spectrometric data was acquired with a Finnigan TSQ7000 triple-stage-quadrupole instrument fitted with an APCI API2 probe and interfaced with an APCI/ESI controller unit. The vaporizer and heated capillary temperatures were maintained at 450°C and 150°C, respectively, and the corona voltage was set at 4 kV. The nitrogen sheath gas pressure was 60 psi; however, the auxiliary gas function was not used. The low mass (0–2,000 amu) range was selected. For recording data in the fullscan mode, the mass spectrometer was scanned from 100 to 650 amu with a scan time of 0.5 s. For MS/MS analyses, the argon gas pressure in the collision cell was set to 2.6 mTorr. Quantitative analyses were carried out in the selected reaction monitoring mode, with the following transitions being monitored: *m*/*z* 433 to *m*/*z* 373 for SA; *m*/*z* 436 to *m*/*z* 373 for SB; *m*/*z* 436 to *m*/*z* 376 for salvinorin A-d_3; and *m*/*z* 439 to *m*/*z* 376 for salvinorin B-d_3. The resolution was set to 0.6 amu and the scan times to 0.25 s. The collision energy for each transition was 15 V.

Measurement of Resting State Electroencephalography (EEG)

Three minutes of resting EEG was obtained immediately following SA inhalation. Subjects sat still with eyes closed to reduce blink and movement artifacts. EEG data were collected in an acoustically shielded booth, and recording was done with the commercially available ActiveTwo acquisition system (Biosemi, the Netherlands). A sampling rate of 1024 Hz

was utilized, with on-line low-pass filter of 256 Hz to prevent aliasing of high frequencies. A 64-channel electrode cap according to the extended 10-20 system was used, along with additional electrodes to record the vertical and horizontal electrooculogram. All electrodes were referenced during recording to a common-mode signal electrode between POz and PO3 and then subsequently re-referenced to the nose offline.

The primary psychophysiological dependent measure was spectral power in each of the traditional EEG frequency bands (delta, 0.1-3 Hz; theta, 4-7 Hz; alpha, 8-12 Hz; beta, 13-29 Hz; gamma, 30-80 Hz). The 3 min of resting-state EEG data was segmented into 2 sec epochs, and artifact rejection included elimination of any epoch ± 100 microvolts. Spectral power was then determined using a Fast Fourier Transform (FFT). The FFT was performed on each epoch, and the data were subsequently averaged. Mean power data for each subject and drug condition were then exported in each frequency band for further statistical analysis. All EEG data were processed and analyzed using the commercially available software package Analyzer 2.0 (Brain Products, Germany).

Supplemental References

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