Behavioral Effects and Central Nervous System Levels of the Broadly Available κ -Agonist Hallucinogen Salvinorin A Are Affected by P-Glycoprotein Modulation In Vivo

Eduardo R. Butelman, Michael Caspers, Kimberly M. Lovell, Mary Jeanne Kreek, and Thomas E. Prisinzano

Laboratory on the Biology of Addictive Diseases, The Rockefeller University, New York, New York (E.R.B., M.J.K.); and Department of Medicinal Chemistry, University of Kansas School of Pharmacy, Lawrence, Kansas (M.C., K.M.L., T.E.P.)

Received February 16, 2012; accepted March 19, 2012

ABSTRACT

Active blood-brain barrier mechanisms, such as the major efflux transporter P-glycoprotein (mdr1), modulate the in vivo/central nervous system (CNS) effects of many pharmacological agents, whether they are used for nonmedical reasons or in pharmacotherapy. The powerful, widely available hallucinogen salvinorin A (from the plant *Salvia divinorum*) is a high-efficacy, selective κ -opioid agonist and displays fast-onset behavioral effects (e.g., within 1 min of administration) and relatively short duration of action. In vitro studies suggest that salvinorin A may be a P-glycoprotein substrate; thus, the functional status of P-glycoprotein may influence the behavioral effects of salvinorin A or its residence in CNS after parenteral administration. We therefore studied whether a competing P-glycoprotein substrate (the clinically available agent loperamide; 0.032–0.32 mg/kg) or a selective P-glycoprotein blocker, tariquidar (0.32–3.2 mg/kg) could enhance unconditioned behavioral effects

(ptosis and facial relaxation, known to be caused by κ -agonists in nonhuman primates) of salvinorin A, as well as its entry and residence in the CNS, as measured by cerebrospinal fluid sampling. Pretreatment with either loperamide or tariquidar dose-dependently enhanced salvinorin A-induced ptosis, but not facial relaxation. In a control study, loperamide and tariquidar were inactive when given as a pretreatment to ((+)-(5 α ,7 α ,8 β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U69,593), a κ -agonist known to be a very poor P-glycoprotein substrate. Furthermore, pretreatment with tariquidar (3.2 mg/kg) also enhanced peak levels of salvinorin A in cerebrospinal fluid after intravenous administration. These are the first studies in vivo showing the sensitivity of salvinorin A effects to modulation by the P-glycoprotein transporter, a major functional component of the blood-brain barrier.

Introduction

Salvinorin A, a high-efficacy, selective κ -opioid receptor agonist, is the main active component of the widely available and powerful hallucinogenic plant *Salvia divinorum* (Roth et al., 2002; Chavkin et al., 2004; Wang et al., 2005). This plant was originally used in ethnomedical practice in Mexico (Oaxaca), but has in recent years seen rapid “decontextualization” and diffusion in the United States with sales in “head shops” and on the internet for recreational/hallucinogenic purposes. Salvinorin A-containing products are at this time under legal

restrictions by laws in various states in the United States and by other countries.

S. divinorum users in naturalistic settings demonstrate behavioral effects with very rapid onsets of effect after inhalation (within 2 min) (Lange et al., 2010). An experimental study in humans inhaling salvinorin A also reported very rapid onsets (with peak effects within 2 min of administration) (Johnson et al., 2011). Variability in subject sensitivity and potential vulnerability to untoward effects of salvinorin A-containing products has also been reported (Singh, 2007; Przekop and Lee, 2009; Siebert, 2010; Johnson et al., 2011). Studies in nonhuman primates have evaluated the detailed time course of unconditioned salvinorin A effects and characterized a set that parallels effects seen in humans (i.e., rapid onset and entry into the CNS) (Butelman et al., 2009).

Of broader impact to translational CNS pharmacology, recent in vitro data suggest that salvinorin A may be a

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grants DA017369 (to E.R.B.), DA018151 (to T.E.P.), DA05130 (to M.J.K.)].

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.

<http://dx.doi.org/10.1124/jpet.112.193227>.

ABBREVIATIONS: CNS, central nervous system; BBB, blood-brain barrier; PET, positron emission tomography; CSF, cerebrospinal fluid; LC/MS/MS, liquid chromatography/tandem mass spectrometry; ANOVA, analysis of variance; PT, pretreatment; U69,593, ((+)-(5 α ,7 α ,8 β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide).

substrate of the major blood-brain barrier efflux transporter, P-glycoprotein (mdr-1, from the *ABCB1* gene). This transporter limits brain concentrations of many pharmacological agents and their therapeutic efficacy, including chemotherapeutic agents for brain cancer, certain antiviral agents used in HIV highly active antiretroviral therapy, and neuropsychiatric/antiepileptic agents (Lin and Yamazaki, 2003; Robey et al., 2010; Moons et al., 2011; Namanja et al., 2012; O'Brien et al., 2012). Nonhuman primates (macaques) may be of particular translational value in this setting, given the reported functional or genetic similarities between macaque and human targets under study, P-glycoprotein and κ -receptors (Liu-Chen, 2004; Butelman et al., 2007; Zolnerciks et al., 2011).

A PET study in nonhuman primates showed that ^{18}F -salvinorin A entered the CNS very rapidly (within 1 min), but also exited very rapidly, consistent with an active efflux mechanism (Hooker et al., 2008). κ -Receptor occupancy of this radiotracer could not be confirmed in the above study, because of possible radionuclide cleavage from the salvinorin A scaffold or other factors. The purpose of the present studies was to provide direct in vivo evidence that salvinorin A behavioral effects and entry into the CNS are sensitive to P-glycoprotein modulation.

Materials and Methods

Subjects. The subjects were adult, gonadally intact, captive-bred rhesus monkeys (four males and three females; 10–16 years old; 7–12.5 kg). They were singly housed in stable colony rooms maintained at 20 to 22°C with controlled humidity and a 12-h light/dark cycle (lights on at 7:00 AM). Subjects were extensively habituated to sit in custom-made aluminum/polycarbonate chairs (chairing occurred with the “pole and collar” method) within a quiet procedure room adjacent to colony rooms.

Subjects were fed appropriate amounts of primate chow biscuits (PMI Feeds, Richmond, VA) daily, supplemented by treats. An environmental enrichment plan was in place in the colony rooms. Water was freely available in home cage via an automatic waterspout. Consecutive experiments in the same subjects in the behavioral assay were typically separated by at least 72 h and separated in the CSF assay by at least 7 days. All experiments were carried out at least 3 h after lights on and 3 h before lights off (10:00 AM–4:00 PM) on each experimental day.

Studies were reviewed and approved by the Rockefeller University Animal Care and Use Committee, in accordance with the *Guide for the Care and Use of Animals* (Institute of Laboratory Animal Resources, 1996).

Unconditioned Behavioral Effects of Salvinatorin A: Ptois and Facial Relaxation. These behavioral endpoints were chosen based on prior studies in which they were used to characterize salvinorin A and, in particular, its rapid-onset (Butelman et al., 2009). Effects of salvinorin A in these endpoints can also be blocked by the opioid antagonist nalmeffene, at doses consistent with mediation by κ -receptors (Butelman et al., 2009). It is also known that a peripherally selective κ -agonist exhibits low potency and low effectiveness in these behavioral endpoints, compared with its profile in a neuroendocrine endpoint thought to be mediated by κ -receptors outside of the BBB (Butelman et al., 1999, 2010).

As described previously (Butelman et al., 2009), the cumulative duration of two specific behaviors was separately quantified in videotaped chaired subjects in 1-min time windows: ptosis (eye closure; partial or complete) and facial relaxation. Thus, scores for each behavior in a time window ranged from 0 to 60 s. Very brief events (<1 s in duration) were not scored to avoid baseline behaviors such

as blinking. Salvinorin A and synthetic κ -agonists cause rapid-onset, robust, and dose-dependent ptosis and facial relaxation, at doses that also result in unresponsiveness to environmental stimuli, an operational measure of sedation (Butelman et al., 2009; see also relevant human data in Ur et al., 1997). An analysis of humans under the influence of *S. divinorum* in a naturalistic setting revealed qualitatively similar effects, supporting translational relevance for these dependent variables (Lange et al., 2010). Time windows (60 s in duration) were rated “blind” by a trained rater using the Observer XT System (Noldus, Wageningen, The Netherlands). In a rater retraining determination, scoring of a sample time window with intermediate ptosis levels was repeated five times by the same observer. The mean ptosis score of these five determinations was 29.4 s (S.E.M. 1.6); therefore, the coefficient of variation was 5.4%, similar to that observed in a prior study using this technique (Butelman et al., 2009).

Each session was composed of a preinjection baseline period, followed by intravenous vehicle or salvinorin A injection, followed by a 60-min observation period. Standard 1-min time windows were scored as 0 to 1, 1 to 2, 4 to 5, 14 to 15, 29 to 30, and 59 to 60 min after administration. These windows were chosen a priori, based on previous studies (Butelman et al., 2009).

Design for Behavioral Studies. The effects of a small and intermediate dose of salvinorin A (0.0032 and 0.01 mg/kg i.v.) were compared with vehicle. We have shown previously that larger salvinorin A doses (0.032 mg/kg) cause near-maximal effects on these endpoints (facial relaxation and ptosis) (Butelman et al., 2009). Given the present focus on the potential ability of P-glycoprotein inhibition in enhancing salvinorin A effects, such larger doses were not studied herein, to avoid interference by “ceiling” effects. The effects of 5-min pretreatment with the P-glycoprotein substrate loperamide (0.032 and 0.32 mg/kg) before salvinorin A (0.01 mg/kg) were studied. As a control, the effects of 5-min loperamide pretreatment (0.32 mg/kg) were studied before a κ -agonist known to be a poor P-glycoprotein substrate [((+)-(5 α ,7 α ,8 β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U69,593); 0.0056 mg/kg i.v.] (Dagenais et al., 2004), as were the effects of loperamide (0.32 mg/kg i.v.) alone. The effects of 30-min pretreatment with the P-glycoprotein blocker tariquidar (0.32 or 3.2 mg/kg) before salvinorin A (0.01 mg/kg) were also studied. As above, the effects of 30-min pretreatment with tariquidar (3.2 mg/kg) on the effects of U69,593 (0.0056 mg/kg i.v.) were studied to determine the specificity of tariquidar effects on a κ -agonist known to be a poor P-glycoprotein substrate (Dagenais et al., 2004). This probe dose of U69,593 was selected because of its similarity in effectiveness to salvinorin A (0.01 mg/kg); larger doses of U69,593 are known to cause robust effects on these endpoints (Butelman et al., 2009).

Effects of the P-Glycoprotein Blocker Tariquidar on Salvinorin A Levels in Cerebrospinal Fluid. Four subjects were fasted overnight and anesthetized with tiletamine/zolazepam (Telazol; Fort Dodge Laboratories, Fort Dodge, IA) (3 mg/kg i.m.). The area around the occiput and upper dorsal neck was clipped, and skin was disinfected with sequential isopropanol and iodine swabs. The subject was placed on a heating pad (37°C). A spinal needle (22 g, 1.5 inches; BD Biosciences, San Jose, CA) was inserted in the cisterna magna as described previously (Lipman et al., 1988). After flow of clear CSF was confirmed, the needle's sterile stylet was replaced into the needle before the study and between samples. This stylet essentially occludes the lumen of the needle up to its tip, minimizing sample cross-contamination. Samples (approximately 300 μl each) were collected in chilled Eppendorf tubes and then placed on dry ice. CSF samples were collected preinjection and at 0, 1, 2, 5, 15, and 30 min after the end of salvinorin A injection (0.01 mg/kg i.v. injected over approximately 20 s). The first drop of CSF at each time point (approximately 50 μl) was not collected to further minimize the cross-contamination of consecutive samples. After the 30-min time point, subjects were allowed to recover under observation and then returned to the home cage. Plasma samples were not analyzed, because

salvinorin A systemic concentrations decay very rapidly over the first minute after intravenous administration (Schmidt et al., 2005; Hooker et al., 2008). Salvinorin A was studied alone or after 30-min pretreatment with tariquidar (3.2 mg/kg i.v., injected over 3 min approximately). Consecutive sessions in the same subject were separated by at least 1 week.

Design of CSF Studies. Based on the parameters of behavioral studies described above, the effects of 30-min tariquidar pretreatment (3.2 mg/kg i.v.) before salvinorin A (0.01 mg/kg) were studied. This tariquidar dose (3.2 mg/kg) is in the range of doses previously found to block P-glycoprotein in PET studies in macaques (Kurdziel et al., 2003; Zoghbi et al., 2008) and is similar to doses used in human PET studies.

LC/MS/MS Analysis of CSF Samples. The salvinorin A concentrations in the CSF samples were determined as follows. CSF samples (stored at -80°C) were thawed on ice until free of any frozen particulates. To a high-recovery sample vial was then added 300 µl of CSF from each time point and diluted to 350 µl, such that the final concentration was 10% (v/v) acetonitrile and 1% (v/v) formic acid and contained 1.0 ng of (2*S*,4*aR*,6*aR*,7*R*,9*S*,10*aS*,10*bR*)-methyl 9-acetoxy-2-(furan-3-carbonyl)-6*a*,10*b*-dimethyl-4,10-dioxododecahydro-1*H*-benzo[*f*]isochromene-7-carboxylate as an internal standard. The samples were then analyzed by LC/MS/MS using multiple reaction monitoring. Samples were separated on a Micro-Tech Scientific (Vista, CA) 1-mm i.d. × 5-cm Zorbax C18 300 Å 3.5-µm column with a 1-mm × 2-cm, 300-Å, 5-µm C18 guard column (Agilent Technologies, Santa Clara, CA) on a Waters (Milford, MA) Acquity UPLC autosampler running under high-performance liquid chromatography conditions and analyzed on a Micromass Quattro Ultima mass spectrometer (Micromass Ltd., Manchester, UK). The high-performance liquid chromatographer was operated in gradient mode

TABLE 1
Gradient elution used during LC/MS/MS analysis
Solvent A was composed of 99.0% H₂O and 1.0% acetonitrile. Solvent B was composed of 99.92% acetonitrile and 0.08% formic acid.

Time	Flow Rate	Solvent A	Solvent B
	µl/min	%	
Initial	0.135	99.0	1.0
1.0 min	0.135	99.0	1.0
2.0 min	0.135	71.0	29.0
10.0 min	0.135	67.0	33.0
10.1 min	0.145	5.0	95.0
11.0 min	0.145	5.0	95.0
12.0 min	0.130	99.0	1.0

Fig. 1. Effects of salvinorin A (0.0032–0.01 mg/kg, iv.) on ptosis (left) and facial relaxation (right). Ordinates, duration of each behavior, within a 60-s time window (mean ± S.E.M.). Abscissae, time from salvinorin A administration (min).

as outlined in Table 1. The data were collected and processed by using MassLynx version 4.1 (Waters). All calculations were based on peak area ratios between salvinorin A and (2*S*,4*aR*,6*aR*,7*R*,9*S*,10*aS*,10*bR*)-methyl 9-acetoxy-2-(furan-3-carbonyl)-6*a*,10*b*-dimethyl-4,10-dioxododecahydro-1*H*-benzo[*f*]isochromene-7-carboxylate, and sample concentrations were determined from the linear range of a standard curve (see Fig. 5).

Data Analysis. All studies included four to five subjects. All data are presented as mean ± S.E.M. and were analyzed with one- or two-way repeated-measures ANOVAs, followed by post hoc tests, using Sigmapstat (SPSS Inc., Chicago, IL) or Prism (GraphPad Software, Inc., San Diego, CA); the α level was set at $p < 0.05$. ANOVA values were presented to two decimal places. Behavioral scores were measured to the nearest 0.1 s. The last data point sample (30 min after salvinorin A) could not be collected in one subject in the tariquidar pretreatment condition because of a lack of CSF backflow. This single data point was replaced by an unbiased estimate (the mean of the three other subjects at this time point, past the observed peak).

Pharmacological Agents. Salvinorin A (extracted in the laboratory of Dr. T. E. Prisinzano, University of Kansas College of Pharmacy, Lawrence, KS) was dissolved daily in ethanol/Tween 80/sterile water [1:1:8 (v/v)]. U69,593 (Pharmacia and Upjohn, Kalamazoo, MI) was dissolved in sterile water and acidified with lactic acid to pH ≈ 5. Loperamide HCl (Sigma, St. Louis, MO), was dissolved in ethanol/Tween 80/sterile water [1:1:8 (v/v)]. Tariquidar (also known as XR9576) was synthesized in the laboratory of Dr. T. E. Prisinzano, using published methods (Bankstahl et al., 2011), and dissolved in dimethyl sulfoxide/Tween80/sterile water [1/1/8 (v/v)]. Doses of each compound are expressed in the forms described above. Appropriate salvinorin A vehicle injections and volumes were used for comparisons in behavioral studies.

Results

Preinjection Baseline, Effects of Vehicle, and Salvinorin A Doses. As expected, preinjection baseline scores on these variables (ptosis and facial relaxation) yielded principally scores of 0 (of a possible 60-s maximum). Likewise, intravenous vehicle administration also resulted in primarily scores of 0 throughout the standard time windows over the 60-min experiment. By contrast, administration of relatively small doses of salvinorin A (0.0032 and 0.01 mg/kg) resulted in active (i.e., nonzero) scores in all animals, with a relatively fast onset (Fig. 1). A two-way [time × dose (vehicle or salvinorin dose)] repeated-measures ANOVA for ptosis yielded a

significant time $\times$ dose interaction ($F_{10,30} = 3.83$; $p < 0.01$). In post hoc Newman-Keuls tests, the larger salvinorin A dose, but not the smaller one, was significantly different from vehicle at the 1- to 2- and 4- to 5-min time windows ($p < 0.01$). The same analysis on facial relaxation data did not show significant main effects or interaction (data not shown).

Effects of the P-Glycoprotein Substrate Loperamide on Salvinorin A-Induced Ptosis and Facial Relaxation.

The P-glycoprotein substrate loperamide, at the largest dose studied (0.32 mg/kg), alone had no effect on ptosis or facial relaxation over a standard 60-min session ($n = 4$; data not shown). Loperamide (0.032 or 0.32 mg/kg i.v.; $n = 4$) was administered as a 5-min pretreatment before salvinorin A (0.01 mg/kg). Loperamide caused a dose-dependent enhancement of the effects of salvinorin A on ptosis (Fig. 2). A two-way (pretreatment condition $\times$ time) repeated-measures ANOVA yielded a significant main effect of pretreatment condition ($F_{2,6} = 17.50$; $p < 0.01$) and a time $\times$ pretreatment interaction ($F_{10,30} = 4.65$; $p < 0.01$). Newman-Keuls tests indicate that the larger, but not the smaller, loperamide dose produced a significant enhancement of salvinorin A-induced ptosis at the 0- to 1-, 1- to 2-, and 4- to 5-min time windows. By contrast, loperamide was devoid of effect on salvinorin

A-induced facial relaxation under the same conditions (data not shown).

In a control study, the larger loperamide dose was administered as a 5-min pretreatment before U69,593, a κ -agonist known to be a poor substrate for P-glycoprotein (Dagenais et al., 2004). The dose of U69,593 (0.0056 mg/kg, i.v.), was selected from published dose-ranging data in this assay and produced peak effects of approximately the same magnitude as salvinorin A (0.01 mg/kg; Fig. 1) (Butelman et al., 2007, 2009). Loperamide under these conditions did not enhance either U69,593-induced ptosis (Fig. 3, left) or facial relaxation (data not shown).

Effects of the P-Glycoprotein Blocker Tariquidar on Salvinorin A-Induced Ptosis and Facial Relaxation.

The P-glycoprotein blocker tariquidar (3.2 mg/kg) alone had no discernible effect on ptosis or facial relaxation as measured 20 min after its injection (before salvinorin A injection; data not shown). Complete control studies with tariquidar alone were therefore not carried out to conserve the supply of this compound. Tariquidar (0.32 or 3.2 mg/kg i.v.; $n = 5$) was administered as a 30-min pretreatment before salvinorin A (0.01 mg/kg). Tariquidar caused a dose-dependent increase in salvinorin A-induced ptosis (Fig. 4), but not facial relaxation (data not shown). A two-way (pretreatment condition $\times$ time) repeated-measures ANOVA yielded significant main effects of time ($F_{5,20} = 8.35$; $p < 0.01$) and pretreatment condition ($F_{2,8} = 7.90$; $p < 0.02$). Newman-Keuls tests indicated that the larger, but not the smaller, tariquidar dose produced a significant enhancement of salvinorin A-induced ptosis ($q = 5.08$; $p < 0.05$). To monitor the duration of action of the larger tariquidar dose (3.2 mg/kg), a separate condition was probed with tariquidar pretreatment 48 h before salvinorin A (0.01 mg/kg; $n = 4$), under identical experimental conditions (data not shown). The enhancing effects of tariquidar had dissipated by this time.

As in the control study above, the larger dose of tariquidar (3.2 mg/kg) did not cause an enhancement in the effects of U69,593 (0.0056 mg/kg), a κ -agonist known to be a poor P-glycoprotein substrate (Fig. 3).

Effects of the P-Glycoprotein Blocker Tariquidar on CSF Levels of Salvinorin A. A standard curve for the detection of salvinorin A in the present assay is presented (Fig. 5). CSF baseline samples, whether in the absence or presence of tariquidar (3.2 mg/kg), had no signal at the mass peak for salvinorin A (i.e., 0 ng/ml), as expected. Salvinorin A

Fig. 2. Effects of 5-min pretreatment with loperamide (0.032–0.32 mg/kg i.v.) on salvinorin A (0.01 mg/kg i.v.)-induced ptosis. Other details are as in Fig. 1.

Fig. 3. Effects of 5-min pretreatment with loperamide (left) or 30-min pretreatment with tariquidar (right) on U69,593 (0.0056 mg/kg, i.v.)-induced ptosis. Other details are as in Fig. 1.

Fig. 4. Effects of 30-min pretreatment with tariquidar (0.32–3.2 mg/kg) on salvinorin A (0.01 mg/kg)-induced ptoxis. Other details are as in Fig. 1.

(0.01 mg/kg i.v.; $n = 4$) had a consistent profile of CSF levels after injection with detectable salvinorin A levels in each subject even immediately after the end of intravenous injection (time 0 min). Peak salvinorin A concentrations were observed at 1 and 2 min after the end of intravenous administration (mean was 1.3 ng/ml in each case, and S.E.M. were 0.4 and 0.3, respectively) (Fig. 6). By the last time point taken (30 min), salvinorin A concentrations had declined to <25% of peak values (i.e., mean was 0.3 ng/ml; S.E.M. was 0.04) (Fig. 5). Follow-up studies at further time points were therefore not instituted. Pretreatment with tariquidar (3.2 mg/kg i.v.) yielded a similar time profile of salvinorin A CSF levels (peak levels were observed at 2 min postinjection), but higher concentrations were detected (Fig. 5). Thus peak levels of salvinorin A (2 min after administration) after tariquidar had a mean of 2.5 ng/ml (S.E.M. 0.3), that is a $\approx 90\%$ increase over values observed after salvinorin A alone. A two-way time $\times$ condition repeated-measures ANOVA yielded a significant main effect of time ($F_{5,15} = 12.96$; $p < 0.01$) and a significant interaction of time $\times$ condition ($F_{5,15} = 3.01$; $p < 0.05$). Newman-Keuls tests indicated that tariquidar (3.2 mg/kg) elevated salvinorin A CSF concentrations at 2 and 5 min after

Fig. 6. Effects of 30-min pretreatment with tariquidar (3.2 mg/kg) on CSF levels of salvinorin A (0.01 mg/kg) administration ($n = 4$). Ordinate, salvinorin A CSF concentration (ng/ml). Abscissa, time from the end of salvinorin A injection (min), where time 0 is immediately after the end of injection.

salvinorin administration ($q = 5.41$ and 4.02 , respectively; $p < 0.05$).

Discussion

This is the first report showing that behavioral effects of salvinorin A, and its levels in the CNS, are enhanced by the presence of a competing P-glycoprotein substrate (loperamide; a compound with peripherally selective μ -agonist effects, because of its sensitivity to P-glycoprotein-mediated BBB efflux) or by a selective P-glycoprotein blocker (tariquidar). These findings are therefore consistent with in vitro studies showing that salvinorin A is a P-glycoprotein substrate (Teksin et al., 2009) and suggest that its effects in self-exposed humans will also depend on the functional status of the P-glycoprotein transporter, which can be affected by genotype or by the presence or chronic exposure to another substrate (Doran et al., 2005; Levran et al., 2008). It may be hypothesized that interindividual sensitivity and vulnerability to untoward effects of salvinorin A in humans may depend at least in part on such factors (Singh, 2007; Przekop and Lee, 2009; Siebert, 2010). The present effects of loperamide and tariquidar on salvinorin A were likely to be mediated by P-glycoprotein mechanisms rather than a more general interaction with the κ -opioid system, because neither loperamide nor tariquidar affected the effects of U69,593, a κ -agonist known to be a poor P-glycoprotein substrate (Dagenais et al., 2004).

The selected dose ranges for loperamide and tariquidar were consistent with studies that focused on their pharmacodynamic profile in humans or nonhuman primates, including PET studies of P-glycoprotein function (Butelman et al., 2008; Wagner et al., 2009; Bauer et al., 2010; Kreisl et al., 2010). The similarity of these conditions is supportive of the translational value of these simple behavioral and CNS penetration measures (see below) as endpoints to study P-glycoprotein modulation in vivo.

Consistent with prior findings in nonhuman primates (Butelman et al., 2009), salvinorin A exhibited very fast onset (within 1–2 min of intravenous injection) of unconditioned behavioral effects (especially ptoxis, at the relatively low

Fig. 5. A representative nine-point LC-MS calibration curve used to quantify salvinorin A in CSF samples. The regression was linear from 0.125 to 50 ng/ml.

doses used herein). A similar profile of fast-onset behavioral effects are observable in humans (Lange et al., 2010; Johnson et al., 2011) and can therefore be considered valuable for translational comparisons. The use of these simple unconditioned behaviors is especially useful in the investigation of a compound such as salvinorin A, where other measures (e.g., operant effects or real-time self-reports in humans) may be impractical or unattainable because of its prominent behavioral obtunding/hallucinogenic profile.

It is of interest that salvinorin A-induced ptosis, but not facial relaxation, was enhanced by loperamide and tariquidar in these studies; this finding was not predicted. Some factors may provide a potential interpretation for further study. First, these simple unconditioned behaviors (ptosis and facial relaxation) are thought to be mediated by different defined brain nuclei, cranial nerve pathways (Wilson-Pauwels et al., 2010). Second, the distribution of P-glycoprotein sites in brain in vivo (in PET assays), and the influence of tariquidar on these sites, is not homogeneous, even among areas traditionally thought to be “inside” the blood-brain barrier (Liow et al., 2009; Bauer et al., 2010; van Assema et al., 2012), although the influence of blood flow differences cannot be wholly discounted. Such neuroanatomically differential effects of P-glycoprotein distribution and function could therefore result in a differential profile of tariquidar-induced enhancement of specific behavioral effects of the proposed P-glycoprotein substrate salvinorin A in these studies (i.e., ptosis versus facial relaxation).

As a caveat, CSF cannot be necessarily considered a direct match of extracellular or parenchymal brain levels of an injected drug; nevertheless CSF levels can be considered a basic biomarker for a drug's residence in CNS tissues in many cases (Lin, 2008). Tariquidar-enhanced peak concentrations of salvinorin A in CSF were observed rapidly (within 2 min of salvinorin A injection), providing some initial insights into the ability of P-glycoprotein to modulate the onset versus duration of action of centrally active drugs, be they drugs of abuse or pharmacotherapeutic agents.

Overall, the present studies provide the first direct in vivo evidence that the behavioral effects and CNS residence of the broadly available hallucinogen salvinorin A are sensitive to the functional status of the major BBB-efflux transporter P-glycoprotein.

Acknowledgments

We thank Szymon Rus for technical assistance.

Authorship Contributions

Participated in research design: Butelman, Kreek, and Prisinzano.

Conducted experiments: Butelman, Caspers, and Prisinzano.

Contributed new reagents or analytic tools: Caspers, Lovell, and Prisinzano.

Performed data analysis: Butelman, Caspers, and Prisinzano.

Wrote or contributed to the writing of the manuscript: Butelman, Caspers, Lovell, Kreek, and Prisinzano.

References

- Bankstahl JP, Bankstahl M, Kuntner C, Stanek J, Wanek T, Meier M, Ding XQ, Müller M, Langer O, and Löscher W (2011) A novel positron emission tomography imaging protocol identifies seizure-induced regional overactivity of P-glycoprotein at the blood-brain barrier. *J Neurosci* **31**:8803–8811.
- Bauer M, Karch R, Neumann F, Wagner CC, Kletter K, Müller M, Löscher W, Zeitlinger M, and Langer O (2010) Assessment of regional differences in tariquidar-induced P-glycoprotein modulation at the human blood-brain barrier. *J Cereb Blood Flow Metab* **30**:510–515.
- Butelman ER, Harris TJ, and Kreek MJ (1999) Effects of E-2078, a stable dynorphin A(1–8) analog, on sedation and serum prolactin levels in rhesus monkeys. *Psychopharmacology (Berl)* **147**:73–80.
- Butelman ER, Mandau M, Tidgewell K, Prisinzano TE, Yuferov V, and Kreek MJ (2007) Effects of salvinorin A, a κ -opioid hallucinogen, on a neuroendocrine biomarker assay in nonhuman primates with high κ -receptor homology to humans. *J Pharmacol Exp Ther* **320**:300–306.
- Butelman ER, Prisinzano TE, Deng H, Rus S, and Kreek MJ (2009) Unconditioned behavioral effects of the powerful κ -opioid hallucinogen salvinorin A in nonhuman primates: fast onset and entry into cerebrospinal fluid. *J Pharmacol Exp Ther* **328**:588–597.
- Butelman ER, Reed B, Chait BT, Mandau M, Yuferov V, and Kreek MJ (2008) Limited effects of β -endorphin compared to loperamide or fentanyl in a neuroendocrine biomarker assay in non-human primates. *Psychoneuroendocrinology* **33**:292–304.
- Butelman ER, Rus S, Prisinzano TE, and Kreek MJ (2010) The discriminative effects of the κ -opioid hallucinogen salvinorin A in nonhuman primates: dissociation from classic hallucinogen effects. *Psychopharmacology (Berl)* **210**:253–262.
- Chavkin C, Sud S, Jin W, Stewart J, Zjawiony JK, Siebert DJ, Toth BA, Hufeisen SJ, and Roth BL (2004) Salvinorin A, an active component of the hallucinogenic sage *Salvia divinorum* is a highly efficacious κ -opioid receptor agonist: structural and functional considerations. *J Pharmacol Exp Ther* **308**:1197–1203.
- Dagenais C, Graff CL, and Pollack GM (2004) Variable modulation of opioid brain uptake by P-glycoprotein in mice. *Biochem Pharmacol* **67**:269–276.
- Doran A, Obach RS, Smith BJ, Hosea NA, Becker S, Callegari E, Chen C, Chen X, Choo E, Cianfrogna J, et al. (2005) The impact of P-glycoprotein on the disposition of drugs targeted for indications of the central nervous system: evaluation using the MDR1A/1B knockout mouse model. *Drug Metab Dispos* **33**:165–174.
- Hooker JM, Xu Y, Schiffer W, Shea C, Carter P, and Fowler JS (2008) Pharmacokinetics of the potent hallucinogen, salvinorin A in primates parallels the rapid onset and short duration of effects in humans. *Neuroimage* **41**:1044–1050.
- Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals* 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington, DC.
- Johnson MW, MacLean KA, Reissig CJ, Prisinzano TE, and Griffiths RR (2011) Human psychopharmacology and dose-effects of salvinorin A, a κ opioid agonist hallucinogen present in the plant *Salvia divinorum*. *Drug Alcohol Depend* **115**:150–155.
- Kreisl WC, Liow JS, Kimura N, Seneca N, Zoghbi SS, Morse CL, Herscovitch P, Pike VW, and Innis RB (2010) P-glycoprotein function at the blood-brain barrier in humans can be quantified with the substrate radiotracer ¹¹C-N-desmethyl-loperamide. *J Nucl Med* **51**:559–566.
- Kurdziel KA, Kiesewetter DO, Carson RE, Eckelman WC, and Herscovitch P (2003) Biodistribution, radiation dose estimates, and in vivo Pgp modulation studies of ¹⁸F-paclitaxel in nonhuman primates. *J Nucl Med* **44**:1330–1339.
- Lange JE, Daniel J, Homer K, Reed MB, and Clapp JD (2010) *Salvia divinorum*: effects and use among YouTube users. *Drug Alcohol Depend* **108**:138–140.
- Levrano O, O'Hara K, Peles E, Li D, Barral S, Ray B, Borg L, Ott J, Adelson M, and Kreek MJ (2008) ABCB1 (MDR1) genetic variants are associated with methadone doses required for effective treatment of heroin dependence. *Hum Mol Genet* **17**:2219–2227.
- Lin JH (2008) CSF as a surrogate for assessing CNS exposure: an industrial perspective. *Curr Drug Metab* **9**:46–59.
- Lin JH and Yamazaki M (2003) Clinical relevance of P-glycoprotein in drug therapy. *Drug Metab Rev* **35**:417–454.
- Liow JS, Kreisl W, Zoghbi SS, Lazarova N, Seneca N, Gladding RL, Taku A, Herscovitch P, Pike VW, and Innis RB (2009) P-glycoprotein function at the blood-brain barrier imaged using ¹¹C-N-desmethyl-loperamide in monkeys. *J Nucl Med* **50**:108–115.
- Lipman B, Palmer D, Noble J, Haughton V, and Collier D (1988) Effect of lumbar puncture on flow of cerebrospinal fluid. *Invest Radiol* **23**:359–360.
- Liu-Chen LY (2004) Agonist-induced regulation and trafficking of κ opioid receptors. *Life Sci* **75**:511–536.
- Moons T, de Roo M, Claes S, and Dom G (2011) Relationship between P-glycoprotein and second-generation antipsychotics. *Pharmacogenomics* **12**:1193–1211.
- Namanja HA, Emmert D, Davis DA, Campos C, Miller DS, Hrycyna CA, and Chmielewski J (2012) Toward eradicating HIV reservoirs in the brain: inhibiting P-glycoprotein at the blood-brain barrier with prodrug abacavir dimers. *J Am Chem Soc* **134**:2976–2980.
- O'Brien FE, Dinan TG, Griffin BT, and Cryan JF (2012) Interactions between antidepressants and P-glycoprotein at the blood-brain barrier: clinical significance of in vitro and in vivo findings. *Br J Pharmacol* **165**:289–312.
- Przekop P and Lee T (2009) Persistent psychosis associated with *Salvia divinorum* use. *Am J Psychiatry* **166**:832.
- Robey RW, Massey PR, Amiri-Kordestani L, and Bates SE (2010) ABC transporters: unvalidated therapeutic targets in cancer and the CNS. *Anticancer Agents Med Chem* **10**:625–633.
- Roth BL, Baner K, Westkaemper R, Siebert D, Rice KC, Steinberg S, Ernsberger P, and Rothman RB (2002) Salvinorin A: a potent naturally occurring nonnitrogenous κ opioid selective agonist. *Proc Natl Acad Sci U S A* **99**:11934–11939.
- Schmidt MD, Schmidt MS, Butelman ER, Harding WW, Tidgewell K, Murry DJ, Kreek MJ, and Prisinzano TE (2005) Pharmacokinetics of the plant-derived κ -opioid hallucinogen salvinorin A in nonhuman primates. *Synapse* **58**:208–210.
- Siebert DJ (2010) Localization of salvinorin A and related compounds in glandular trichomes of the psychoactive sage, *Salvia divinorum*. *Ann Bot* **93**:763–771.
- Singh S (2007) Adolescent salvia substance abuse. *Addiction* **102**:823–824.
- Teksin ZS, Lee LJ, Nemieboka NN, Othman AA, Upreti VV, Hassan HE, Syed SS, Prisinzano TE, and Eddington ND (2009) Evaluation of the transport, in vitro metabolism and pharmacokinetics of salvinorin A, a potent hallucinogen. *Eur J Pharm Biopharm* **72**:471–477.

- Ur E, Wright DM, Bouloux PM, and Grossman A (1997) The effects of spiradoline (U-62066E), a κ -opioid receptor agonist, on neuroendocrine function in man. *Br J Pharmacol* **120**:781–784.
- van Assema DM, Lubberink M, Bauer M, van der Flier WM, Schuit RC, Windhorst AD, Comans EF, Hoetjes NJ, Tolboom N, Langer O, et al. (2012) Blood-brain barrier P-glycoprotein function in Alzheimer's disease. *Brain* **135**:181–189.
- Wagner CC, Bauer M, Karch R, Feurstein T, Kopp S, Chiba P, Kletter K, Löscher W, Müller M, Zeitlinger M, et al. (2009) A pilot study to assess the efficacy of tariquidar to inhibit P-glycoprotein at the human blood-brain barrier with (R)-11C-verapamil and PET. *J Nucl Med* **50**:1954–1961.
- Wang Y, Tang K, Inan S, Siebert D, Holzgrabe U, Lee DY, Huang P, Li JG, Cowan A, and Liu-Chen LY (2005) Comparison of pharmacological activities of three distinct κ ligands (Salvinorin A, TRK-820 and 3FLB) on κ opioid receptors in vitro and their antipruritic and antinociceptive activities in vivo. *J Pharmacol Exp Ther* **312**:220–230.
- Wilson-Pauwels L, Stewart PA, Akesson EJ, and Spacey SD (2010) *Cranial Nerves: Function and Dysfunction*. People's Medical Publishing House, Shelton, CT.
- Zoghbi SS, Liow JS, Yasuno F, Hong J, Tuan E, Lazarova N, Gladding RL, Pike VW, and Innis RB (2008) 11C-loperamide and its *N*-desmethyl radiometabolite are avid substrates for brain permeability-glycoprotein efflux. *J Nucl Med* **49**:649–656.
- Zolnericiks JK, Booth-Genthe CL, Gupta A, Harris J, and Unadkat JD (2011) Substrate- and species-dependent inhibition of P-glycoprotein-mediated transport: implications for predicting in vivo drug interactions. *J Pharm Sci* **100**:3055–3061.

Address correspondence to: E. R. Butelman, The Rockefeller University, Box 171, 1230 York Avenue, New York, NY 10065. E-mail: butelme@rockefeller.edu