Behavioral effects and CNS levels of the broadly available κ-agonist hallucinogen salvinorin A are affected by p-glycoprotein modulation in vivo

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BBB: blood-brain barrier; p-gp: p-glycoprotein; PT: pre-treatment

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Abstract

Active blood-brain barrier mechanisms, such as the major efflux transporter p-glycoprotein (mdr1) modulate the in vivo/CNS effects of many pharmacological agents, be they used for non-medical 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 minute of administration), and relatively short duration of action. In vitro studies suggest that salvinorin A may be a p-glycoprotein substrate, and thus the functional status of p-glycoprotein may influence the behavioral effects of salvinorin A, or its residence in CNS following 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 non-human primates) of salvinorin A, as well as its entry and residence in 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 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, following its i.v. administration. These are the first studies in vivo showing 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.

*Salvia divinorum* users in naturalistic settings demonstrate behavioral effects with very rapid onsets of effect after inhalation (e.g., within 2 min) (Lange et al., 2010). An experimental study in humans inhaling salvinorin A also reported very rapid onsets (e.g., 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 non-human primates have evaluated the detailed time course of unconditioned salvinorin A effects, and have characterized a set that parallel effects seen in humans (i.e., rapid onset and entry into CNS) (Butelman et al., 2009).

Of broader impact to translational CNS pharmacology, recent *in vitro* data suggest that salvinorin A may be a 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 anti-viral agents used in HIV HAART and neuropsychiatric/antiepileptic agents (Lin and Yamazaki, 2003; Robey et al., 2010; Moons et al., 2011; Namanja et al., 2011; O’Brien et al., 2011). Non-human 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 non-human primates showed that 18F-salvinorin A entered the CNS very rapidly (within 1 min), but also exited very rapidly, consistent with active efflux mechanism (Hooker et al., 2008). κ-receptor occupancy of this radiotracer could not be confirmed in the above, due to possible radionuclide cleavage from the salvinorin A scaffold, or other factors. The purpose of the present studies was to provide the first direct in vivo evidence that salvinorin A behavioral effects, and entry into CNS are sensitive to p-glycoprotein modulation.
Methods

Subjects: The subjects were adult, gonadally intact, captive bred rhesus monkeys (Four male and three female; age range, 10–16 years old; weight range 7–12.5 kg). They were singly housed in stable colony rooms maintained at 20-22°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 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 (e.g., 1000–1600 hours) 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 (National Academy Press, 1996; Washington, DC).

Unconditioned behavioral effects of salvinorin A – Ptosis and facial relaxation. These behavioral endpoints were chosen for study herein based on prior studies in which they were used to characterize salvinorin A, and in particular its rapid-onset effects (Butelman et al., 2009). Effects of salvinorin A in these endpoints can also be blocked by the opioid antagonist nalmefene, at nalmefene 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 to its profile in a neuroendocrine endpoint thought to be mediated by κ-receptors outside the BBB (Butelman et al., 1999; Butelman et al., 2010).
As recently described (Butelman et al., 2009), the cumulative duration of two specific behaviors were 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 sec. Very brief events (e.g., <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 (Ur et al., 1997). An analysis of humans under the influence of Salvia divinorum in a naturalistic setting, revealed qualitatively similar effects, supporting translational relevance for these dependent variables (Lange et al., 2010).

Time windows (60 sec in duration) were rated “blind” by a trained rater using the Observer XT System (Noldus, Wageningen, The Netherlands). In a recent rater re-training 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 sec (SEM 1.6), therefore the coefficient of variation was 5.4%, similar to that observed in a prior publication with this technique (Butelman et al., 2009).

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

**Design for behavioral studies:** The effects of a small and intermediate dose of i.v. salvinorin A (0.0032 mg/kg and 0.01 mg/kg) were compared to vehicle. We have recently shown that larger salvinorin A doses (e.g., 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, in order 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) prior to salvinorin A (0.01 mg/kg) were studied. As a control, the effects of 5 min loperamide pretreatment (0.32 mg/kg) were studied prior to a κ-agonist known to be a poor p-glycoprotein substrate (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) prior to 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 also 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 due to 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 (CSF). Four subjects were fasted overnight and were anesthetized with Telazol (3 mg/kg i.m.). The area around the occiput and upper dorsal neck was clipped, and skin 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 previously described (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 were then placed on dry ice. CSF samples were collected preinjection and at the following times after the end of salvinorin A injection (0.01 mg/kg i.v. injected over approximately 20 s): 0, 1, 2, 5, 15, and 30 min. The first drop of CSF at each time point (approximately 50 μl) was not collected, in order to further minimize cross-contamination of consecutive samples. After the 30 min time point, subjects were allowed to recover under observation and were then returned to
the home cage. Plasma samples were not analyzed herein, since we have previously found that salvinorin A systemic concentrations decay very rapidly over the 1st min after i.v. 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.) prior to 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% acetonitrile, 1% formic acid, and contained 1.0 ng of (2S,4aR,6aR,7R,9S,10aS,10bR)-methyl 9-acetoxy-2-(furan-3-carbonyl)-6a,10b-dimethyl-4,10-dioxododecahydro-1H-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 1mm ID×5 cm Zorbax C18 300 Å 3.5 micron column with a 2 cm, 300 Å 5 micron C18 guard column on a Waters Acquity UPLC autosampler running under HPLC conditions, and analyzed on a Micromass Quatro Ultima mass spectrometer. The HPLC was operated in gradient mode as outlined in Table 1. The data was collected and processed using MassLynx version 4.1 (Waters Inc.). All calculations were based on peak area ratios between salvinorin A and (2S,4aR,6aR,7R,9S,10aS,10bR)-methyl 9-acetoxy-2-(furan-3-carbonyl)-
6a,10b-dimethyl-4,10-dioxododecahydro-1\textsuperscript{H}benzo[f]isochromene-7-carboxylate and sample concentrations were determined from the linear range of a standard curve (see Figure 5).

**Data Analysis:** All studies are n=4-5, within subjects. All data are presented as mean ±SEM; and were analyzed with 1 or 2-way repeated measures ANOVAs, followed by post-hoc tests (SPSS Sigmastat or Graphpad Prism); the $\alpha$ level was set at p<0.05. ANOVA values were presented to 2 decimal places. Behavioral scores were measured to the nearest 0.1 sec. The last data point sample (30 min post-salvinorin A) could not be collected in one subject in the tariquidar pretreatment condition, due to 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 ((+)-(5α,7α,8β)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide; 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 was dissolved in DMSO/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 thereof were used for comparisons in behavioral studies.
Results

Pre-injection baseline, effects of vehicle, and salvinorin A doses: As expected, pre-injection baseline scores on these variables (ptosis and facial relaxation) yielded principally scores of 0 (out of a possible 60 sec 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., non-zero) scores in all animals, with a relatively fast onset (Fig. 1). A two-way (time X dose [vehicle or salvinorin dose]) repeated measures ANOVA for ptosis, yielded a significant time X 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 were significantly different from vehicle at the 1-2 and 4-5 min time windows (p<0.01). The same analysis on facial relaxation data did not show significant main effects or interaction (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; not shown). Loperamide (0.032 or 0.32 mg/kg i.v., n=4) was administered as a 5 min pretreatment prior to salvinorin A (0.01 mg/kg). Loperamide caused a dose-dependent enhancement of the effects of salvinorin A on ptosis (Fig. 2). A 2-way (pretreatment condition X time) repeated measures ANOVA yielded a significant main effect of pretreatment condition (F[2,6]=17.50; p<0.01), and a time X 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 0-1, 1-2 and 4-5 min time windows. By contrast, loperamide was devoid of effect on salvinorin A-induced facial relaxation, under the same conditions (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; see Fig. 1) (Butelman et al., 2007; Butelman et al., 2009). Loperamide, under these conditions did not enhance either U69,593- induced ptosis (Fig. 3; left panel) or facial relaxation (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 (i.e., prior to salvinorin A injection; not shown). Complete control studies with tariquidar alone were therefore not carried out, to conserve 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 (not shown). A 2-way (pretreatment condition X 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 indicate that the larger, but not the smaller, tariquidar dose produced a significant enhancement of salvinorin A-induced ptosis (q=5.08; p<0.05). In order to monitor the duration of action of the larger tariquidar dose (3.2 mg/kg), a separate condition was probed with tariquidar pretreatment 48h before salvinorin A (0.01 mg/kg; n=4), under identical experimental conditions (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 (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 i.v. injection (time “0” min). Peak salvinorin A concentrations were observed at 1 and 2 min after the end of i.v. administration (mean was 1.3 ng/ml in each case, and SEM 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; SEM 0.04) (Fig. 5). Follow-up studies at further time points were therefore not instituted herein. Pretreatment with tariquidar (3.2 mg/kg i.v.) yielded a similar time profile of salvinorin A CSF levels (e.g., peak levels were observed at 2 min post-injection), 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 (SEM 0.3), that is a ≈90% increase over values observed after salvinorin A alone. A 2-way time X 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 X 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 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 CNS, are enhanced by the presence of a competing p-glycoprotein substrate (loperamide; a compound peripherally selective μ-agonist effects, due to it 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 presence or chronic exposure to another substrate) (Doran et al., 2005; Levran et al., 2008). It may be hypothesized that inter-individual 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 non-human 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 non-human primates (Butelman et al., 2009), salvinorin A exhibited very fast onset (within 1-2 minutes of i.v. injection) of unconditioned behavioral effects (especially ptosis, at the relatively low 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 due to its prominent behavioral obtunding/hallucinogenic profile.

It is of interest that salvinorin A induced ptosis, but not facial relaxation, were 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 (e.g., 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., 2011), although 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 vs. 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). Interestingly, tariquidar-enhanced peak concentrations of salvinorin A in CSF were still observed rapidly (within 2 min of salvinorin A injection), providing some initial insights into the ability of p-glycoprotein to modulate the onset vs. 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 is sensitive to the functional status of the major BBB-efflux transporter, p-glycoprotein.
Acknowledgments:

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**Authorship Contributions**

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

*Conducted experiments:* Butelman, Caspers, Prisinzano.

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

*Performed data analysis:* Butelman, Caspers, Prisinzano.

*Wrote or contributed to the writing of the manuscript:* All authors
References


Legends for Figures

Figure 1. Effects of salvinorin A (0.0032-0.01 mg/kg, iv.) on ptosis and facial relaxation (left and right panels, respectively. Ordinates: Duration of each behavior, within a 60-sec time window (mean ±SEM). Abscissae: Time from salvinorin A administration (min).

Figure 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 as in Fig. 1.

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

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

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

Figure 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.
Table 1: Gradient elution used during LC/MS/MS analysis.

<table>
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<tr>
<th>Time (min)</th>
<th>Flow Rate (μL/min)</th>
<th>% Solvent A&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% Solvent B&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup>Solvent A was composed of 99.0% H₂O and 1.0% acetonitrile.

<sup>b</sup>Solvent B was composed of 99.92% acetonitrile and 0.08% formic acid.
Figure 1
Figure 2

Graph showing the effect of salvinorin A (0.01 mg/kg) alone and in combination with loperamide (0.032 mg/kg PT and 0.32 mg/kg PT) on ptosis duration over time.

- **Circle**: Salvinorin A (0.01 mg/kg) alone
- **Square**: + Loperamide 0.032 mg/kg PT
- **Triangle**: + Loperamide 0.32 mg/kg PT
Figure 3
Figure 4

![Graph showing the effect of salvinorin A (0.01 mg/kg) alone and with Tariquidar on ptosis in mice.](image-url)

- **salvinorin A (0.01 mg/kg) alone**
- **+Tariquidar 0.32 PT**
- **+Tariquidar 3.2 PT**

**Y-axis:** Ptosis (sec)

**X-axis:** Time from salvinorin A administration (min)
Figure 5

\[ y = 3679.9x + 781.37 \]

\[ R^2 = 0.9993 \]
Figure 6

![Graph showing CSF salvinorin A levels over time]

- **salvinorin A 0.01 mg/kg**
- **tariquidar 3.2 mg/kg PT to salvinorin A 0.01 mg/kg**

CSF salvinorin A (ng/ml) vs. Time from the end of salvinorin A i.v. injection (min), n=4