

# Effects of salvinorin A, a $\kappa$ -opioid hallucinogen, on a neuroendocrine biomarker assay in non-human primates with high $\kappa$ -receptor homology to humans

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## Abstract

This study focused on the *in vivo* effects of the  $\kappa$ -opioid hallucinogen salvinorin A, derived from the plant *Salvia divinorum*. The effects of salvinorin A (0.0032-0.056 mg/kg; i.v.) were studied in a neuroendocrine biomarker assay of the anterior pituitary hormone prolactin in gonadally intact, adult male and female rhesus monkeys (N=4 each). Salvinorin A produced dose- and time-dependent neuroendocrine effects, similar to the synthetic high efficacy  $\kappa$ -agonist U69,593, but of shorter duration than the latter. Salvinorin A was approximately equipotent to U69,593 in this endpoint (salvinorin A ED<sub>50</sub>=0.015 mg/kg; U69,593 ED<sub>50</sub>=0.0098 mg/kg). The effects of i.v. salvinorin A were not prevented by a small dose of the opioid antagonist nalmefene (0.01 mg/kg; s.c.) but were prevented by a larger dose of nalmefene (0.1 mg/kg); the latter nalmefene dose is sufficient to produce  $\kappa$ -antagonist effects in this species. By contrast, the 5HT<sub>2</sub> receptor antagonist ketanserin (0.1 mg/kg; i.m.) did not prevent the effects of salvinorin A. As expected, the neuroendocrine effects of salvinorin A (0.0032 mg/kg; i.v.) were more robust in female than in male subjects. Related studies focused on full length cloning of the coding region of the rhesus monkey  $\kappa$ -opioid receptor (*OPRK1*) gene, and revealed a high homology of this non-human primate *OPRK1* gene compared to the human *OPRK1* gene, including particular C-terminal residues thought to be involved in receptor desensitization and internalization. The present studies indicate that the hallucinogen salvinorin A acts as a high efficacy  $\kappa$ -agonist in non-human primates, in a translationally viable neuroendocrine biomarker assay.

## Introduction

Salvinorin A, a diterpenoid, is the main active compound from the leaves of the hallucinogenic plant, *Salvia divinorum* (Valdes, 1994). *Salvia divinorum* preparations were originally used in ethnomedical practice by the Mazatec people of Oaxaca, Mexico, but have recently become widely commercially available. There are emerging reports of use of such salvinorin A –containing products as hallucinogens, mainly by the smoking route (Baggott et al., 2004; Gonzales et al., 2006).

A recent study determined that salvinorin A was a highly selective agonist at  $\kappa$ -opioid receptors (Roth et al., 2002). Salvinorin A was approximately equipotent and equieffective *in vitro* to arylacetamide  $\kappa$ -agonists such as U69,593, in the stimulation of GTP $\gamma$ S binding, or the inhibition of adenylate cyclase (Roth et al., 2002). In another signal transduction system (potentiation of GIRK-channel currents), salvinorin A appeared to be an “ultra-high” efficacy agonist (Chavkin et al., 2004). Salvinorin A was also found to have a lesser propensity to cause  $\kappa$ -receptor desensitization and internalization *in vitro*, compared to arylacetamide  $\kappa$ -agonists (Wang et al., 2004). It is unknown whether this *in vitro* profile confers salvinorin A with unique properties as a  $\kappa$ -agonist *in vivo*, possibly underlying its hallucinogenic effects.

There are some studies of the effects of salvinorin A *in vivo*, mostly in rodents. Salvinorin A caused  $\kappa$ -receptor mediated place aversion and decreases in striatal dopamine dialysates in mice, similarly to synthetic  $\kappa$ -agonists (Zhang et al., 2004; Zhang et al., 2005). Salvinorin A also caused depressive-like behavioral effects and reduced dopamine dialysate levels in N. Accumbens, in rats (Carlezon et al., 2006). Salvinorin A produced  $\kappa$ -receptor mediated sedation / motor incoordination in mice (Fantegrossi et al., 2005). Salvinorin A may produce brief antinociceptive effects under certain conditions in rodents, but is devoid of anti-pruritic effects, typically observed with  $\kappa$ -agonists (Ko et al., 2003; Wang et al., 2004; McCurdy et al., 2006; Ansonoff et al., 2006). Salvinorin A was generalized by non-human primates trained to discriminate U69,593 in an operant assay (Butelman et al., 2004). Few

studies have addressed *in vivo* the apparent efficacy of salvinorin A, or that have endpoints that may be easily adapted to humans, thus having translational value.

Serum prolactin levels have been used in non-human primates to study the potency, receptor selectivity and apparent efficacy of  $\kappa$ -agonists *in vivo* (other compounds, including  $\mu$ -opioid agonists, also cause prolactin release) (Bowen et al., 2002; Butelman et al., 2002). This neuroendocrine biomarker assay has also been used in clinical populations in the study of  $\kappa$ -opioid effects of the neuropeptide dynorphin A (Kreek et al., 1999; Bart et al., 2003). These studies document the effects of salvinorin A in this biomarker assay, and are consistent with the high efficacy ascribed to salvinorin A at  $\kappa$ -receptors, based on *in vitro* studies.

Non-human primates, such as *Macaca mulatta* (used herein) may be valuable models for translational studies of  $\kappa$ -opioid function. Studies suggest that there are differences in rodent vs. human or non-human primate  $\kappa$ -receptor populations, in terms of neuroanatomical localization, relative  $B_{max}$ , and neurobiological interactions (Mansour et al., 1988; Rothman et al., 1992; Berger et al., 2006). Also, comparative studies in cloned human and rat  $\kappa$ -receptors have detected differences in agonist-induced desensitization and internalization, and these could be ascribed to inter-species differences in protein structure at the C-terminus of the receptor (e.g., at the 358 aminoacid residue position; Li et al., 2002; Liu-Chen, 2004). In order to determine whether this non-human primate species shares these critical aminoacid residues with human  $\kappa$ -receptors, we present information on full-length cloning of the coding region of *M. mulatta*  $\kappa$ -receptor.

## Methods

### Methods:

**Experimental subjects in neuroendocrine studies:** Captive-bred, gonadally intact rhesus monkeys (*Macaca mulatta*; 4 male and 4 female; age range: 8-11 years old approximately; weight range: 5.8-12.5 kg), were used. Monkeys were singly housed in a room maintained at 20-22°C with controlled humidity, and a 12:12 hour light: dark cycle (lights on at 0700). Monkeys were fed approximately 11 jumbo primate chow biscuits (PMI Feeds, Richmond, VA) daily, supplemented by appetitive treats, and multivitamins plus iron. An environmental enrichment plan was in place in the colony rooms. Water was freely available in home cages, via an automatic waterspout.

**Procedure for neuroendocrine experiments:** Chair-trained monkeys were tested after extensive prior exposure to the experimental situation. Monkeys were chaired and transferred to the experimental room between 1000h and 1100h on each test day. An indwelling catheter (24 gauge; Angiocath, Becton Dickinson, Sandy, UT) was placed in a superficial leg vein, and secured with elastic tape. An injection port (Terumo, Elkton, MD) was attached to the hub of the catheter; port and catheter were flushed (0.3 ml of 50 U/ml heparinized saline) before use, and after each blood sampling or i.v. injection. Approximately 15 min following catheter placement, two baseline blood samples of approximately 2 ml were collected, 5 min apart from each other (defined as -10 and -5 min, relative to the onset of dosing), and kept at room temperature until the time of spinning (3,000 rpm at 4°C) and serum separation. Serum samples were then kept at -40°C until the time of analysis; typically within 2 weeks of collection. The samples were analyzed in duplicate with a standard human prolactin immunoradiometric kit (DPC, Los Angeles CA), following manufacturer's instructions. There is high protein homology between human and rhesus monkey prolactin, and antibody cross-reactivity between human and rhesus monkey prolactin has also been reported (Brown and Bethea, 1994; Pecins-Thompson et al., 1996; Ordog et al., 1998). The reported sensitivity limit of the present assay was 0.1 ng/ml; each individual kit was calibrated with known

standards, in the range 2-200 ng/ml. The intra- and inter-assay coefficients of variation with this kit in the laboratory were 2% and 9%, respectively.

Monkeys were tested in a time course procedure. Following baseline sample collection, a single agonist (salvinorin A or U69,593) injection was administered, followed by sampling at 5, 15, 30, 60, 90 and 120 min after administration. Unless otherwise stated, agonists were injected by the i.v. route. In antagonism experiments, a single dose of antagonist (s.c. nalmefene or i.m. ketanserin) was administered 30 min before salvinorin A, followed by testing as above. Each experiment was typically carried out in 4 males; selected experiments were carried out in 4 females in follicular phase (days 2-12 of each cycle of approximately 28 days, as defined by the onset of visible bleeding). Consecutive experiments in the same subject were separated by at least 96 hours.

**Design of neuroendocrine studies:** Time course studies were carried out with salvinorin A and U69,593 (0.0032-0.056 mg/kg i.v.; typically n=4), and vehicle. For salvinorin A and U69,593, the largest dose was only studied in 3 of 4 subjects. The fourth subject was not administered the largest dose for safety reasons, based on greater sensitivity to untoward effects of the compounds (e.g., tremors). In other studies, the opioid antagonist nalmefene (0.01 or 0.1 mg/kg, s.c.) was administered as a pretreatment before the largest salvinorin A dose at which all subjects were studied (0.032 mg/kg). A similar pretreatment study was completed with the 5HT<sub>2</sub> antagonist ketanserin (0.1 mg/kg; i.m.), before salvinorin A (0.032 mg/kg). Female subjects were studied at the 0.0032 mg/kg i.v. dose, a dose which results in robust prolactin elevation in females but not in males. Female subjects were also studied after subcutaneous administration of salvinorin A (0.032 mg/kg), with and without nalmefene (0.1 mg/kg, s.c.) pretreatment.

**Data Analysis:** Prolactin values are presented as mean  $\pm$ SEM, after subtraction of individual mean pre-injection baselines for each session ( $\Delta$ ng/ml). Dose-effect curves are also presented, as collated from a

time of peak prolactin release caused by salvinorin A or U69,593 (15 min post- i.v. injection). Linear regression was used to calculate ED<sub>50</sub> values calculated by linear regression from individual data points above and below the 50% level of effect.

Significant differences in a parameter (e.g., log ED<sub>50</sub> values) were considered to occur if there was a lack of overlap in their 95% confidence limits. Unless otherwise stated, experiments were carried out with n=4. Repeated measures ANOVAs were followed by post-hoc tests (using either Graphpad Prism or SPSS-Sigmastat); the level of significance ( $\alpha$ ) was set at p=0.05.

**Drugs:** Salvinorin A was extracted from commercially obtained *S. divinorum* leaves (Ethnogens.com; Berkeley, CA) in the laboratory of Dr. T.E. Prisinzano, as described previously (Tidgewell et al., 2004; Harding et al., 2006). Briefly, dried *S. divinorum* leaves (1.5 kg) were ground to a fine powder and percolated with acetone. The acetone extract was concentrated under reduced pressure to afford a crude green gum, which was subjected to repeated column chromatography on silica gel with elution, using a mixture of EtOAc/hexanes to afford salvinorin A (TLC) and other minor diterpenes. The melting point, <sup>1</sup>H NMR, and <sup>13</sup>C spectra of salvinorin A were in agreement with previous reports (Ortega et al., 1982; Valdes et al., 1984). Salvinorin A solutions for injection were prepared daily in ethanol:Tween80:Sterile water (1:1:8, v/v; maximum concentration in this vehicle was 0.2 mg/ml).

Nalmefene HCl (Baker Norton, FL) was dissolved in sterile water; U69,593 ((+)-(5 $\alpha$ ,7  $\alpha$ ,8 $\beta$ )-N-methyl-N-[7-(1-pyrrolidiniyl)-1-oxaspiro[4.5]dec-8yl]-benzeneacetamide; Pharmacia-Upjohn, Kalamazoo, MI) was dissolved in sterile water with the addition of 1 drop of lactic acid. All above drug doses are expressed as mg/kg of the aforementioned forms indicated above. Ketanserin tartrate (Sigma, St. Louis, MO) was dissolved in 5% DMSO in sterile water (v/v), and was injected i.m.. The ketanserin dose is expressed as the base, for consistency with prior publications (Fantegrossi et al., 2002). All drugs were injected in volumes of 0.05-0.1 ml/kg, whenever possible.



**Cloning and sequencing of *Macaca mulatta*  $\kappa$ -opioid receptor (*OPRK1*) cDNA:** The coding region of the *OPRK1* gene was obtained by PCR amplification of *Macaca mulatta* brain cDNA (obtained from BioChain, Hayward, CA) with the a forward primer 5'-TCCTCGCC TT CCTGCTGCA-3', located 30 nucleotides upstream of ATG codon, and a reverse primer 5'-TCAGACTGC AGTAGTATC-3', located 69 nucleotides downstream the termination codon. The primer design was based on the human *OPRK1* sequence (GenBank NM\_000912). The final product, approximately 1260 base pairs in size, was purified using QIAquick PCR Purification Kit (QIAGEN, Valencia, CA) and cloned in pCR<sup>®</sup>II plasmid (Invitrogen, Carlsbad, CA). The clones were sequenced in both directions using the Big Dye Terminator Cycle Sequencing Kit (ABI, Applied Biosystems, Foster City, CA) and an ABI Prism 3700 capillary sequencer.

**Single nucleotide polymorphism analysis:** Genomic DNA was isolated from peripheral white blood cells, obtained by venipuncture from 14 subjects in the colony, including all 8 subjects used in the present neuroendocrine studies (Versagene kit; Gentrasystems; Minneapolis, MN). The C-terminal of the *Macaca mulatta* *OPRK1* was amplified using a forward primer 5'-ATTCTCTACGCCTTTCTTGAT-3', located 160 base pairs upstream of the termination codon, and a reverse primer 5'-TCAGACTGCAGTAG TATC-3', located 69 base pairs downstream of the termination codon. PCR products, 257 base pairs in size, were sequenced to identify single nucleotide polymorphisms, as described above.

## Results

### Baseline prolactin values and effects of vehicle administration:

Pre-injection prolactin values in males were relatively consistent and exhibited small decreases over time, after i.v. vehicle administration. Thus, mean pre-injection values were 15.5 ng/ml (SEM=4.2); these values decreased gradually over a 120 min session, following i.v. administration of vehicle (1:1:8 ethanol:Tween 80:sterile water v/v; 0.16 ml/kg); see Fig. 1. Female subjects in follicular phase had similar pre-injection baselines (mean=15.1 ng/ml; SEM=3.0), and also exhibited a gradual decrease in prolactin levels over the 120 min experiment following i.v. vehicle.

### Effects of Salvinatorin A or U69,593:

#### Male subjects.

Intravenous salvinatorin A and U69,593 (0.0032-0.056 mg/kg) caused robust dose- and time-dependent increases in prolactin levels (Fig. 1). Salvinatorin A effects were observable by 5 min after i.v. administration, peaked at 15 min after administration, and declined gradually by 120 min. A two-way (time X dose) repeated measures ANOVA for i.v. salvinatorin A (5-120 min and 0.0032-0.032 mg/kg and vehicle) revealed a main effect of time ( $F[5,15]=13.00$ ), dose ( $F[3,9]=12.48$ ) and their interaction ( $F[15,45]=9.70$ ). The largest salvinatorin A dose (0.056 mg/kg) was not included in this analysis because one of the subjects could not be studied at this dose, for safety reasons. Newman-Keuls comparisons at different times post-salvinatorin A revealed significant differences for all salvinatorin A doses (except the smallest dose 0.0032 mg/kg) vs. vehicle at 5, 15 and 30 min. At 60 min, only the largest salvinatorin A (0.032 mg/kg) was different from vehicle. By 90 and 120 min after salvinatorin A, no significant differences were detected with Newman-Keuls comparisons.

U69,593 effects were similar to those of salvinatorin A, with longer duration of action, as suggested by prolactin elevations persisting at the end of the 120 min study period (Fig. 1). A two-way (time X dose) repeated measures ANOVA for i.v. U69,593 (5-120 min and 0.0032-0.032 mg/kg and vehicle) revealed a main effect of time ( $F[5,15]=15.73$ ), dose ( $F[3,9]=32.99$ ), and their interaction ( $F[15,45]=16.10$ ). The

largest U69,593 dose (0.056 mg/kg) was not included in the analysis because one of the subjects could not be tested at this dose (the same subject as in the salvinorin A studies). Newman-Keuls comparisons at different times post U69,593 revealed significant differences for all salvinorin A doses (except the smallest dose, 0.0032 mg/kg) vs. vehicle at 5, 15, 30, 60 and 90 min. At 120 min after U69,593, only the largest U69,593 dose (0.032 mg/kg) was significantly different from vehicle.

Dose-effect curves for salvinorin A and U69,593 were plotted at 15 min after i.v. administration (a time of peak effect), and exhibit approximately equal maximum effect and potency. Clear maximum “plateau” effects were not observed at the largest doses studied in each subject, and this limited the quantitative determination of maximum plateau by non-linear regression. Larger doses than those used herein (i.e. 0.032 for one subject and 0.056 for the other three) were not probed, due primarily to solubility limitations. Intravenous potency was quantified by linear regression, and did not differ significantly between salvinorin A and U69,593 ( $ED_{50}$  for salvinorin A = 0.015 mg/kg [95%CL=0.0048-0.050];  $ED_{50}$  for U69,593 = 0.0098 mg/kg [95%CL=0.0041-0.020]).

**Subcutaneous administration of salvinorin A:** The effects of a probe dose of salvinorin A (0.032 mg/kg) were also studied by the subcutaneous route in male subjects, and resulted in much smaller prolactin release than that observed by the intravenous route, and a slower onset. For example, the peak effect after s.c. salvinorin A (0.032 mg/kg) was observed at 60 min post-injection, and reached a maximum mean of 31.4  $\Delta$ ng/ml (SEM=11.6) (Fig. 3; note y-axis break added for illustration). A one-way repeated measures ANOVA for time (including mean pre-injection baseline and 5-120 min after salvinorin A administration) was significant ( $F[6,18]=7.27$ ). Newman-Keuls comparisons revealed that s.c. salvinorin A 0.032 mg/kg produced a prolactin increase compared to pre-injection baseline only at 60, 90 and 120 min.

**Antagonism experiments:** In separate studies, nalmefene (0.01 or 0.1 mg/kg) was administered as a pretreatment to salvinorin A (0.032 mg/kg, i.v.), a dose that produced maximal or near-maximal prolactin release in all subjects. The smaller nalmefene pretreatment dose did not cause significant antagonism of salvinorin A, whereas the larger nalmefene dose (0.1 mg/kg) robustly antagonized the

effects of salvinorin A (Fig. 4). A two-way repeated measures ANOVA (time X pretreatment condition [no pretreatment vs. nalmefene 0.01 or 0.1 mg/kg]) revealed significant effects of time ( $F[5,15]=11.78$ ) and pretreatment condition ( $F[2,6]=11.49$ ) and their interaction ( $F[10,30]=8.54$ ). Newman-Keuls comparisons revealed that only the larger nalmefene pretreatment dose (0.1 mg/kg) was significantly different from the “no pretreatment” condition. Antagonism surmountability experiments were not attempted for practical reasons; primarily solubility limitations for salvinorin A. In a separate pretreatment study with the 5-HT<sub>2</sub> antagonist ketanserin, (0.1 mg/kg, i.m.), no antagonism of the same probe dose of salvinorin A (0.032 mg/kg; i.v.) was observed (Fig. 4).

**Female subjects:** Salvinorin A (0.0032 mg/kg; i.v.) was studied in follicular phase females (n=4). This salvinorin A dose, which produced only slight effects in males (above), produced larger prolactin elevations for this assay in the female subjects (see Fig. 5; with comparison to male subjects). Pilot studies with larger salvinorin A i.v. doses (0.032 mg/kg) revealed even greater neuroendocrine effects. In order to probe the effects of salvinorin A route of administration, a larger salvinorin A dose (0.032 mg/kg) was studied by the subcutaneous route. In females, s.c. salvinorin A produced robust prolactin release from 15 min after administration, and this effect persisted for at least 120 min (see Fig. 6). This effect of salvinorin A was prevented by nalmefene (0.1 mg/kg; s.c. 30 min pretreatment). In this case also, the effects of savinorin A were more robust in females than in males (compare Figs. 3 and 6).

### **Cloning of rhesus monkey $\kappa$ -opioid receptor gene (*OPRK1*) and genotyping of C-terminal**

**sequence:** The cloned cDNA contained 30 base pairs of the 5'-untranslated region, 1140 base pairs of the coding region, and 87 base pairs of the 3'-untranslated region. The obtained full-length coding sequence for rhesus monkey *OPRK1* was compared to the published sequence for the human *OPRK1*. There were 21 nucleotides and 6 amino acid residues that differed between the rhesus monkey and human *OPRK1* (see Figure 7). Two predicted amino acid residue changes are located in the N-terminal and two others are in the C-terminal of the rhesus monkey *OPRK1*, compared to the human *OPRK1* (Fig

7). Notably, a proposed phosphorylation site serine residue (S358) in the C-terminal in the human *OPRK1* is conserved in the rhesus monkey *OPRK1* (Liu-Chen, 2004). Sequence analysis of the C-terminal of genomic DNA from 14 rhesus monkeys in the colony (including all 8 subjects used in the present neuroendocrine studies; 4 male and 4 female) confirms the presence of this S358 residue in all subjects; no polymorphisms were detected in this region overall.

## Discussion

The main aim of these studies was to examine the neuroendocrine effects of the widely available hallucinogen salvinorin A, in an assay shown to be a useful biomarker for  $\kappa$ -opioid agonist effects in rhesus monkeys. A related aim of these studies was to determine the similarity of the rhesus monkey  $\kappa$ -receptor sequence, given reports of relevant inter-species differences between human  $\kappa$ -receptors and common experimental rodent species such as rat (Liu-Chen, 2004).

Salvinorin A was reported to be a selective  $\kappa$ -agonist, with potentially unique pharmacodynamic effects (Roth et al., 2002; Chavkin et al., 2004; Wang et al., 2004). In the present studies, i.v. salvinorin A caused robust dose-dependent prolactin release in male rhesus monkeys. Salvinorin A was approximately equipotent and equieffective to the synthetic high-efficacy  $\kappa$ -agonist U69,593, similarly to initial *in vitro* reports (Roth et al., 2002). As expected from prior studies in humans, probe experiments with salvinorin A in gonadally intact female monkeys revealed quantitatively greater effects (Kreek et al., 1999). A probe experiment revealed that the neuroendocrine effects of a probe salvinorin A dose (0.032 mg/kg) were significantly greater by the i.v. than the s.c. route in males. Reasons for this are unclear, but may be related to pharmacokinetic factors, possibly limiting bioavailability by the subcutaneous route (Schmidt et al., 2005).

As mentioned above, salvinorin A is a highly selective agonist at  $\kappa$ -receptors; however it is known that other compounds, including  $\mu$ -agonists, can also cause prolactin release in mammals. We therefore carried out antagonism studies with the clinically available compound nalmefene, which acts as a  $\mu$ -opioid antagonist in rhesus monkeys at small doses (e.g., 0.01 mg/kg), and acts as both a  $\mu$ - and  $\kappa$ -antagonist at relatively larger doses (e.g., 0.1 mg/kg) (France and Gerak, 1994; Butelman et al., 2002). In these studies, the smaller dose of nalmefene mentioned above did not prevent the effects of salvinorin A, whereas the larger dose of nalmefene fully prevented such effects. Taken together with previous data (France and Gerak, 1994; Butelman et al., 2002), these studies are consistent with mediation by  $\kappa$ -receptors in the neuroendocrine effects of salvinorin A.

Salvinorin A is distinct from classic hallucinogens such as LSD, in that it does not bind to the 5-HT<sub>2A</sub> receptor (Roth et al., 2002). We wanted to determine whether the present neuroendocrine effects of salvinorin A could be indirectly mediated by 5-HT<sub>2</sub> receptors. In a probe experiment, the 5-HT<sub>2</sub> antagonist ketanserin (0.1 mg/kg) did not block the neuroendocrine effects of salvinorin A under the present conditions. This dose of ketanserin was sufficient to block the reinforcing effects of the stimulant / hallucinogen MDMA (“ecstasy”) in this species (Fantegrossi et al., 2002); MDMA is also known to cause prolactin release in humans (Grob et al., 1996). Overall, this experiment supports the conclusion that salvinorin A produces this neuroendocrine effect through  $\kappa$ -, and not 5-HT<sub>2</sub> receptors in primates.

Cloning of the rhesus monkey *OPRK1* gene revealed greater predicted homology to human  $\kappa$ -receptor (374 of 380 residues; 98.4% homology) compared to that of other experimental species previously reported (see Liu-Chen, 2004, for review). Rhesus monkey *OPRK1*, as determined from cDNA and confirmed by genotyping the present subjects, exhibit the S358 residue in the C-terminal, which is present in human *OPRK1*. Studies indicate that this residue is of critical importance for the maintenance of adaptations including receptor desensitization and internalization (Liu-Chen, 2004). Interestingly, this residue is not conserved in rat *OPRK1*, and this may underlie the lesser propensity for such adaptations in rat *OPRK1 in vitro*. Overall, these initial studies suggest that rhesus monkey *OPRK1* may have greater functional similarity to human *OPRK1* than those of other experimental species. This is the first report, to our knowledge, of full length cloning of a non-human primate  $\kappa$ -receptor. As expected based on studies of  $\mu$ -receptors, non-human primates may provide valuable insights into species differences that may occur with other experimental subjects such as rodents (Miller et al., 2004).

In summary, the widely available hallucinogen salvinorin A produced effects consistent with high efficacy agonist actions at  $\kappa$ -receptors, in a neuroendocrine biomarker of translational value. This confirms the  $\kappa$ -receptor as the primary site of action *in vivo* of this unique hallucinogen. These are, to our knowledge, the first data on the neuroendocrine effects of salvinorin A in any species. Salvinorin A's

effects in this assay are consistent with reports of fast onset and relatively short duration of salvinorin A-containing preparations in humans (Baggott et al., 2004; Gonzales et al., 2006).



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## **Footnotes**

The present studies were reviewed by the Rockefeller University Animal Care and use Committee and the Guide for the Care and Use of Animals (National Academy Press; Washington DC, 1996).

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## Legends for Figures

**Figure 1.** Time course effects of i.v. salvinorin A (left panel) and i.v. U69,593 (right panel) on serum prolactin levels in male subjects (0.0032-0.056 mg/kg; N=4, except at the largest dose, which was N=3). Abscissae: Time in min from i.v. injection. Ordinates: Serum prolactin levels, expressed as change from individual pre-injection baseline ( $\Delta$ ng/ml). Data are mean  $\pm$  SEM; in cases where no error bars are visible, these fall within the symbol for each data point.

**Figure 2.** Dose-effect curve for the effects of i.v. salvinorin A and i.v. U69,593 on serum prolactin levels in male subjects (data are obtained from 15 min after administration of each dose; see Fig. 1). Abscissa: Dose of salvinorin A or U69,593. Ordinate: Serum prolactin levels, expressed as change from individual pre-injection baseline ( $\Delta$ ng/ml). Other details as in Fig. 1.

**Figure 3.** Time course effects of salvinorin A (0.0032 mg/kg) administered by the intravenous or subcutaneous route on serum prolactin levels in male subjects (N=4 each). Abscissa: Time in min from injection. Ordinate: Serum prolactin levels, expressed as change from individual pre-injection baseline ( $\Delta$ ng/ml; note axis break). Other details as in Fig. 1.

**Figure 4.** Effects of nalmefene (0.01 or 0.1 mg/kg; s.c.) or ketanserin (0.1 mg/kg; i.m.) pretreatment to the effects of salvinorin A (0.032 mg/kg; i.v.) in male subjects (N=4 each). Abscissae: Time in min from i.v. injection (point above "N" or "K" are samples obtained 20 min after administration of nalmefene or ketanserin alone, respectively). Ordinates: Serum prolactin levels, expressed as change from individual pre-injection baseline ( $\Delta$ ng/ml). Other details as in Fig. 1.



**Figure 5.** Effects of i.v. salvinorin A (0.0032 mg/kg) or vehicle in male or female subjects (N=4 each).

Other details as in Fig. 1.

**Figure 6.** Effects of subcutaneous salvinorin A (0.032 mg/kg) alone or after pretreatment with nalmeferene (0.1 mg/kg; s.c.) in female subjects. Point above “N” represents sample obtained 20 min after nalmeferene alone. Other details as in Fig. 1.

**Figure 7.** Aminoacid coding sequence for rhesus monkey  $\kappa$ -opioid receptor (*OPRK1*) cloned from cDNA. Rhesus monkey sequence is compared to published sequence for human *OPRK1* (GenBank Accession #NM\_000912), and rat *OPRK1* (GenBank Accession #NM\_017167) (Yakovlev et al., 1995).

Fig. 1

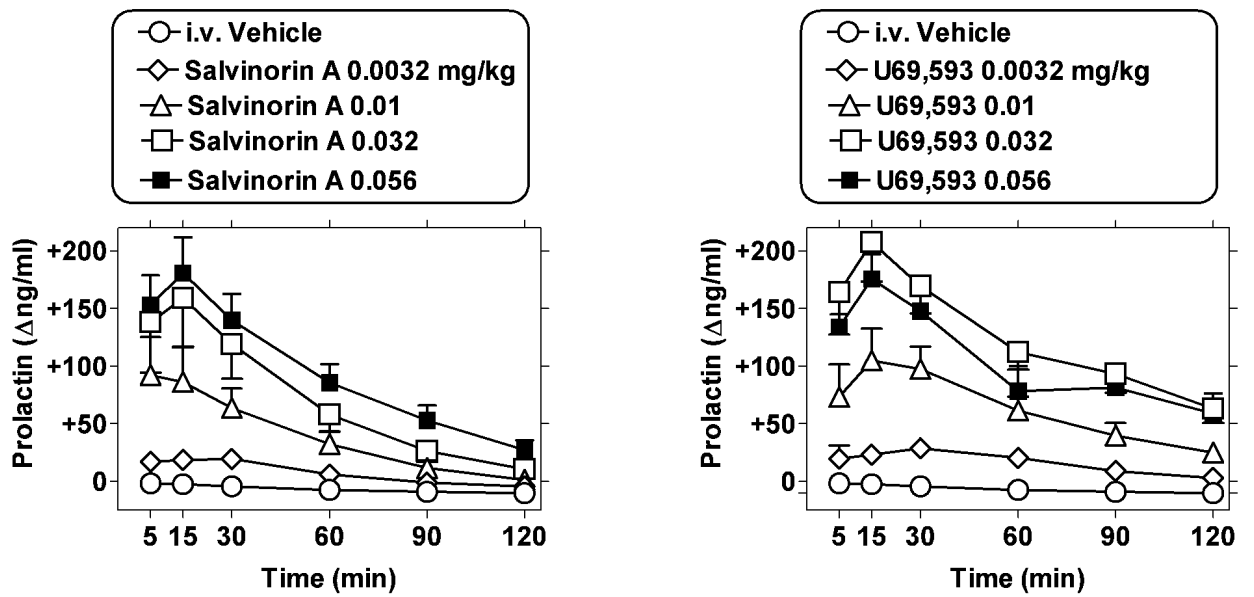


Fig. 2

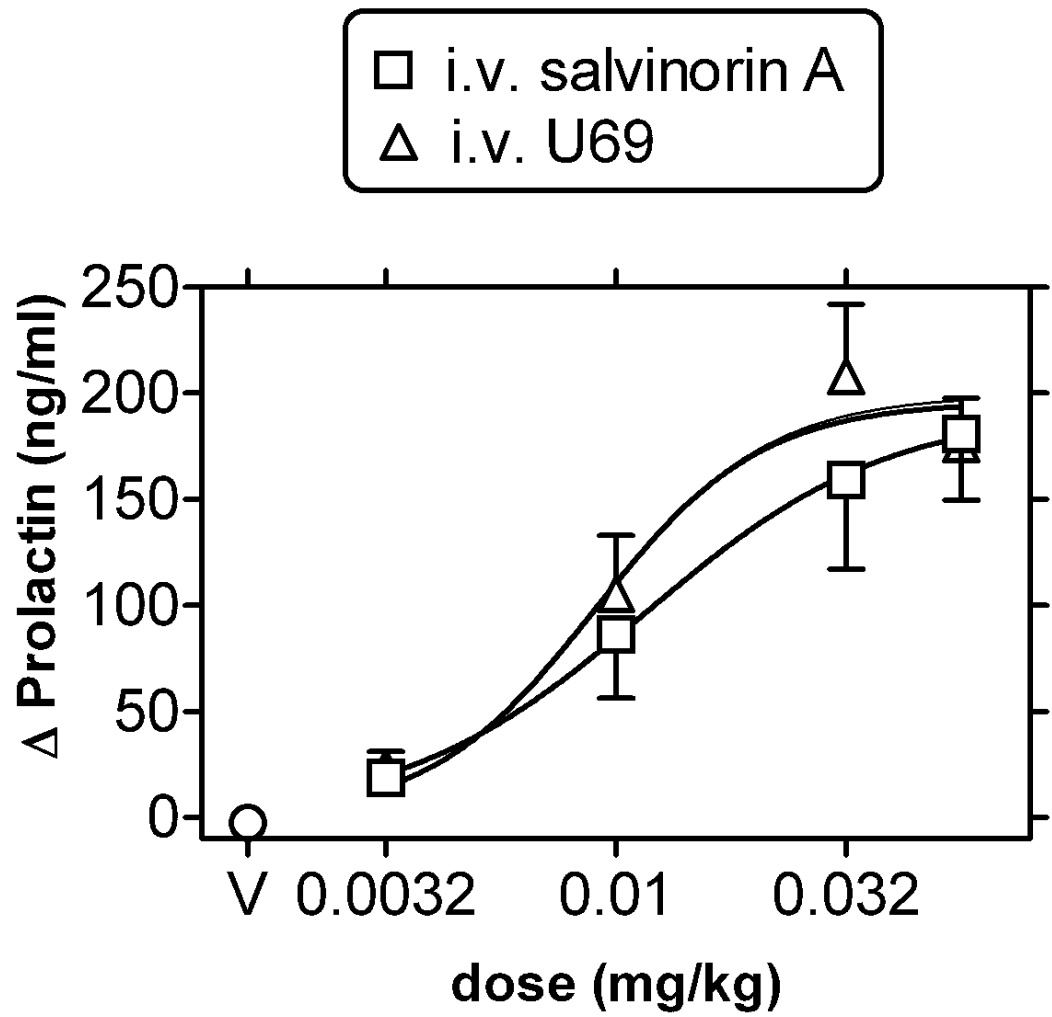


Figure 3.

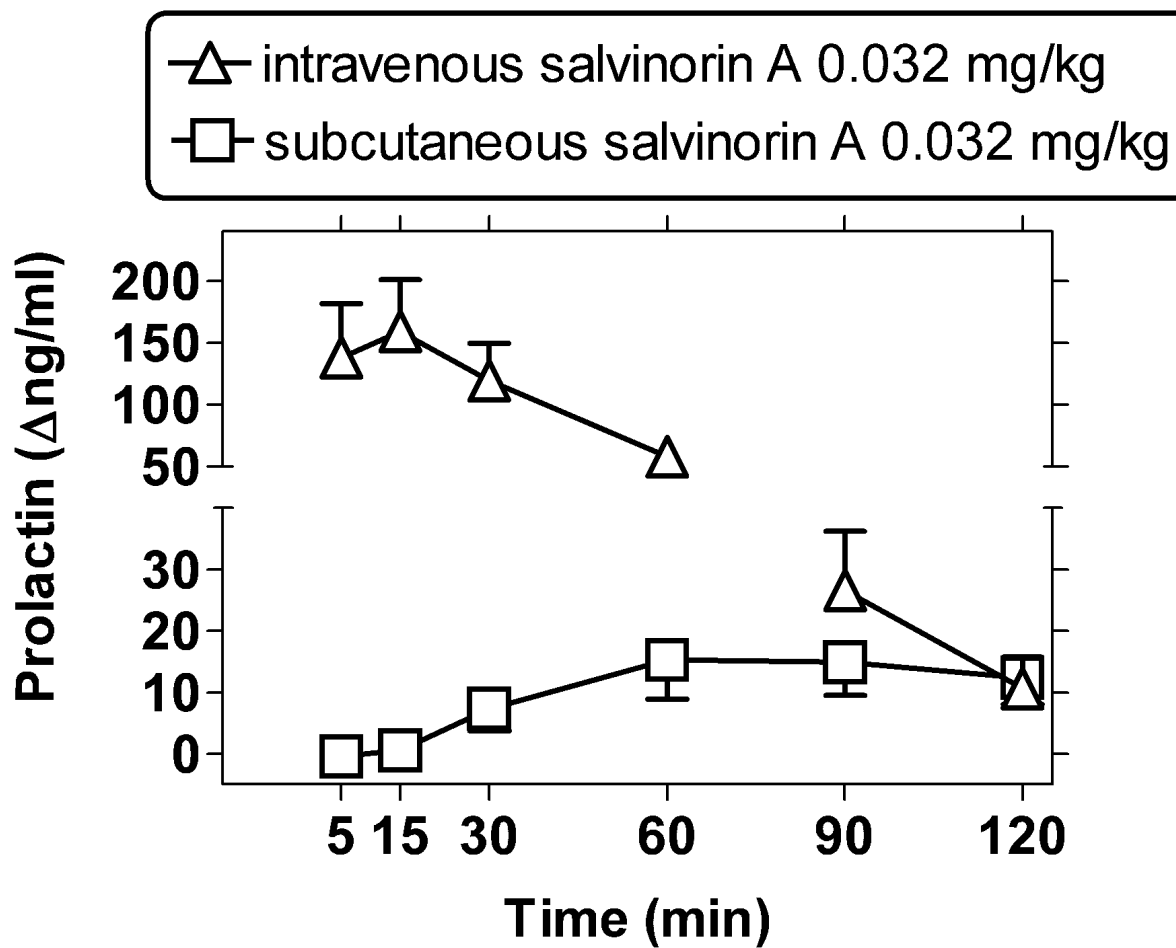


Figure 4.

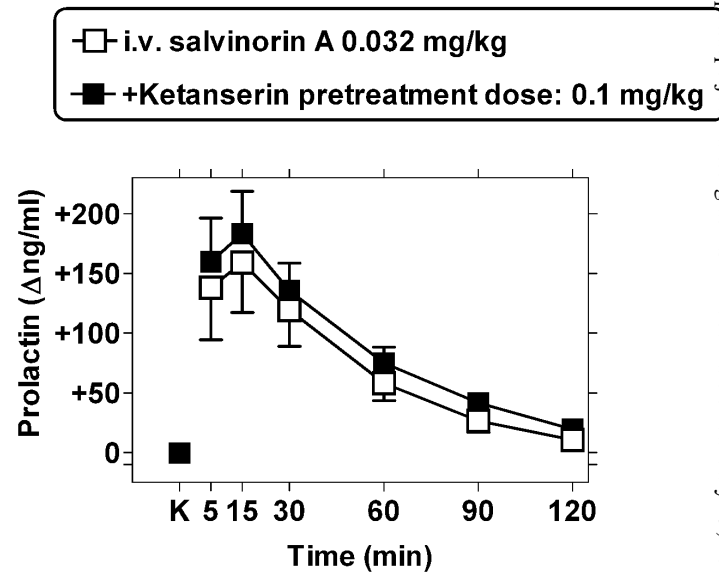
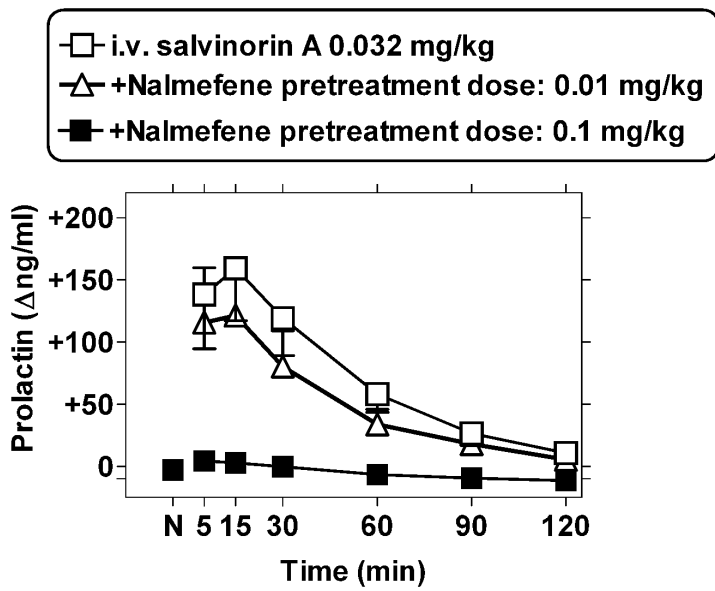


Fig. 5

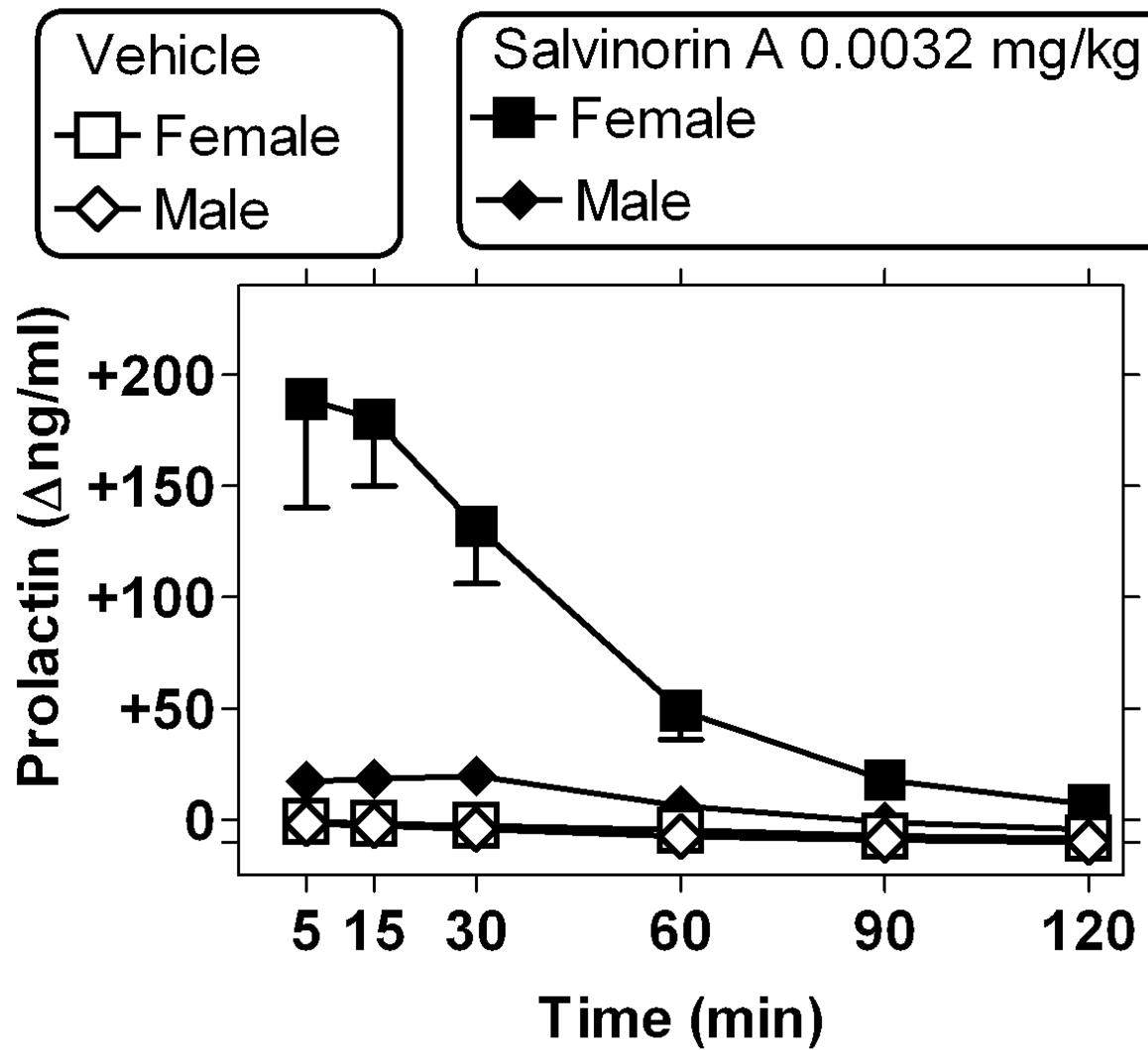
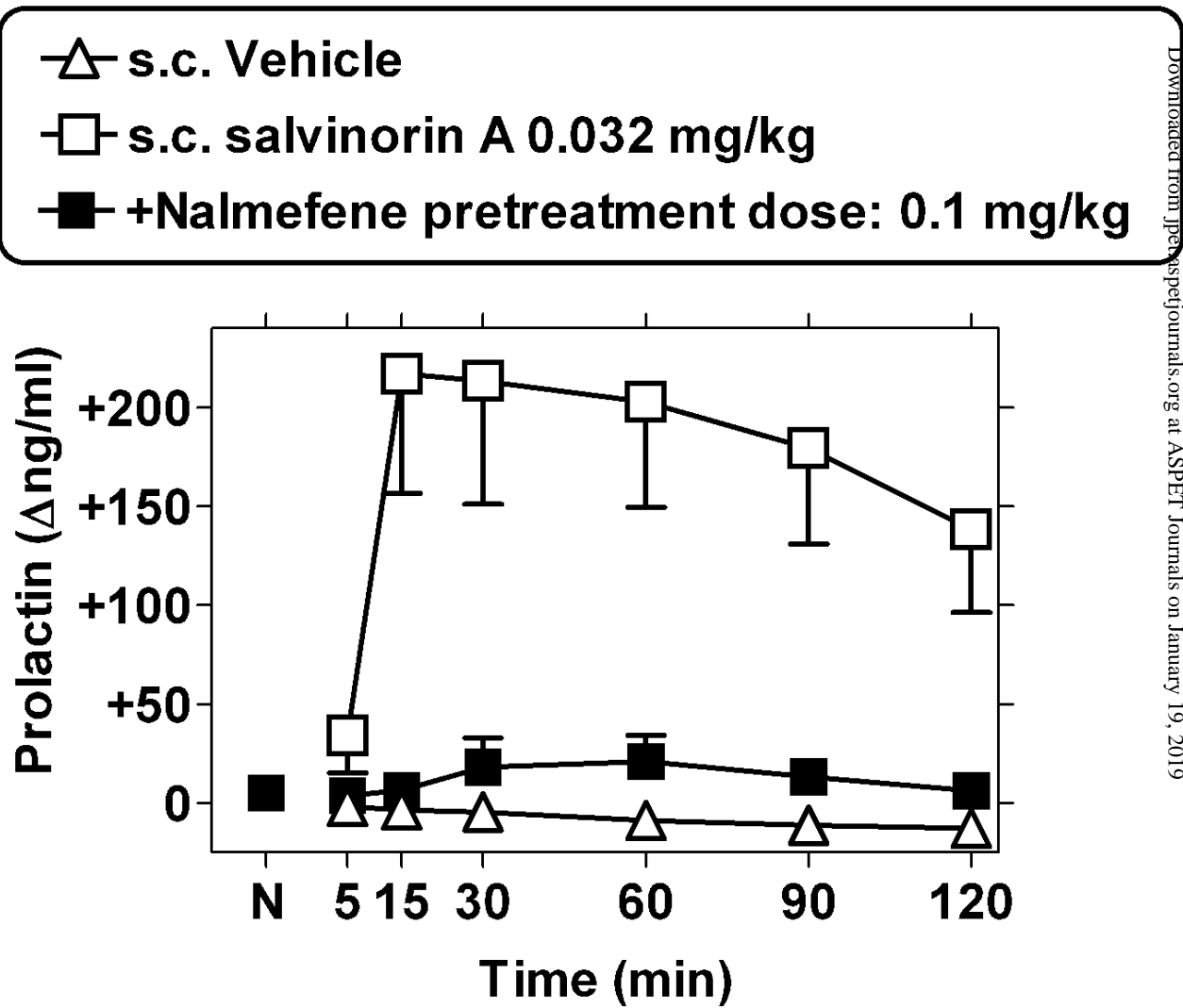


Fig. 6



## Figure 7.

Rhesus OPRK1	1	MDS <b>P</b> VQIFRGEFGPTCAPSACLPPNSSAWFPGWAE <b>L</b> DSNGSAGSEDAQLEPAHISPAIPV	
Human OPRK1	1	MDSPIQIFRGEFGPTCAPSACLPPNSSAWFPGWAE <b>P</b> DSNGSAGSEDAQLEPAHISPAIPV	
Rat OPRK1	1	<b>M</b> ESPIQIFRGEFGPTCAPSACL <b>L</b> PNSS <b>S</b> WFF <b>N</b> WAE <b>S</b> DSNGS <b>V</b> GSE <b>D</b> QLEPAHISPAIPV	
Rhesus OPRK1	61	IITAVYSVVFFVGLVGN <b>S</b> LVMFVIIRYTKMKTATNIYIFNLALADALVTTT <b>M</b> PFQSTVYL	
Human OPRK1	61	IITAVYSVVFFVGLVGN <b>S</b> LVMFVIIRYTKMKTATNIYIFNLALADALVTTT <b>M</b> PFQSTVYL	
Rat OPRK1	61	IITAVYSVVFFVGLVGN <b>S</b> LVMFVIIRYTKMKTATNIYIFNLALADALVTTT <b>M</b> PFQ <b>S</b> AVYL	
Rhesus OPRK1	121	MNSWPF <b>G</b> DVLCKIVISIDYY <b>N</b> MFTSIFTLT <b>M</b> MSVD <b>R</b> YIAVCH <b>P</b> VKALDFRTPLKAKIINI	
Human OPRK1	121	MNSWPF <b>G</b> DVLCKIVISIDYY <b>N</b> MFTSIFTLT <b>M</b> MSVD <b>R</b> YIAVCH <b>P</b> VKALDFRTPLKAKIINI	
Rat OPRK1	121	MNSWPF <b>G</b> DVLCKIVISIDYY <b>N</b> MFTSIFTLT <b>M</b> MSVD <b>R</b> YIAVCH <b>P</b> VKALDFRTPLKAKIINI	
Rhesus OPRK1	181	CIWLLSS <b>S</b> VGISAI <b>V</b> LG <b>G</b> TKVREDVDVIECSLQFPDD <b>D</b> YSW <b>W</b> DLFMKICV <b>F</b> VFAFV <b>I</b> PV <b>L</b>	
Human OPRK1	181	CIWLLSS <b>S</b> VGISAI <b>V</b> LG <b>G</b> TKVREDVDVIECSLQFPDD <b>D</b> YSW <b>W</b> DLFMKICV <b>F</b> I <b>F</b> AFV <b>I</b> PV <b>L</b>	
Rat OPRK1	181	CIWLL <b>A</b> SSVGISAI <b>V</b> LG <b>G</b> TKVREDVDVIECSLQFPDD <b>E</b> YSW <b>W</b> DLFMKICV <b>F</b> VFAFV <b>I</b> PV <b>L</b>	
Rhesus OPRK1	241	IIIVCYTLMILRLK <b>S</b> VRL <b>L</b> SGSREKDRNLRRITRLVLVV <b>V</b> AVF <b>I</b> VCWTP <b>I</b> HI <b>F</b> ILVEALG	
Human OPRK1	241	IIIVCYTLMILRLK <b>S</b> VRL <b>L</b> SGSREKDRNLRRITRLVLVV <b>V</b> AVF <b>V</b> VCWTP <b>I</b> HI <b>F</b> ILVEALG	
Rat OPRK1	241	IIIVCYTLMILRLK <b>S</b> VRL <b>L</b> SGSREKDRNLRRIT <b>K</b> LVLVV <b>V</b> AVF <b>I</b> ICWTP <b>I</b> HI <b>F</b> ILVEALG	
Rhesus OPRK1	301	STSHSTAALSS <b>Y</b> FCIALGYTN <b>S</b> SLNPILYAFLDENFKRCFRDFCFPLK <b>M</b> RMERQSTSRV	
Human OPRK1	301	STSHSTAALSS <b>Y</b> FCIALGYTN <b>S</b> SLNPILYAFLDENFKRCFRDFCFPLK <b>M</b> RMERQSTSRV	
Rat OPRK1	301	STSHSTA <b>V</b> LSS <b>Y</b> FCIALGYTN <b>S</b> SLNP <b>V</b> LYAFLDENFKRCFRDF <b>F</b> Y <b>F</b> IK <b>M</b> RMERQST <b>N</b> RV	
Rhesus OPRK1	361	RNTVQDPAYLRD <b>V</b> D <b>G</b> I <b>N</b> KPV	374/380 Homology vs. human (98.4%)
Human OPRK1	361	RNTVQDPAYLRDIDGM <b>N</b> KPV	
Rat OPRK1	361	RNTVQDP <b>A</b> SMR <b>D</b> V <b>G</b> GM <b>N</b> KPV	360/380 Homology vs. human (94.7%)