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Antinociceptive and hypothermic effects of salvinorin A are abolished in a novel strain of KOR-1 KO mice

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Running Title: Salvinorin A is a κ opioid agonist in vivo

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Abbreviations: salvinorin A (Sal A); salvinorinyl-2-propionate (Sal P); salvinorin B (Sal B); κ-opioid receptors (KORs); intracerebroventricularly (ICV); knockout (KO); U69,593,(+)-(5□,7□,8□)-N-Methyl-N-[7-(1-pyrrolidinyl)-1-oxaspirodec-8-yl]-benzeneacetamide;2-(p-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanatobenzimidazole-HCL(BIT);N-phenyl-N-[1-(2-(p-isothiocyanato)phenylethyl)-4-piperidinyl]propanamide-HCL (FIT); U50,488H, ()-(*trans*

)-3,4-dichloro- *N*-methyl- *N*-[2-(1-pyrrolidiny)-cyclohexyl]benzeneacetamide

Abstract

Salvia divinorum is a natural occurring hallucinogen that is traditionally used by the Mazatec Indians of central Mexico. The diterpene salvinorin A was identified as an active component of *S. divinorum* over 20 years ago but only recently has biochemical screening indicated that a molecular target of salvinorin A *in vitro* is the κ-opioid receptor. We have examined whether salvinorin A, the C2-substituted derivative salvinorinyl-2-propionate, and salvinorin B can act as κ-opioid receptor agonists *in vivo*. We found that following intracerebroventricular injection over a dose range of 1-30 μg both salvinorin A and salvinorinyl-2-propionate produce antinociception in wild-type mice, but not in a novel strain of κ-opioid receptor knockout mice. Moreover, both salvinorin A and salvinorinyl-2-propionate reduce rectal body temperature, similar to conventional κ-opioid receptor agonists, in a genotype-dependent manner. Additionally, we determined that salvinorin A has high affinity for κ_1 but not κ_2 opioid receptors, demonstrating selectivity for this receptor subclass. Finally, treatment over the same dose range with salvinorin B, which is inactive in vitro, produced neither antinociceptive nor hypothermic effects in wild type mice. These data demonstrate that salvinorin A is the active component of *S. divinorum*, selective for κ_1 opioid receptors, and that salvinorin A and specific structurally related analogues produce behavioral effects that require the κ-opioid receptor.

Introduction

Salvia divinorum is a natural occurring hallucinogen that has been used traditionally for divination and other spiritual practices by the Mazatec people of Oaxaca, Mexico (Valdes et al., 1983) and more recently as a legal hallucinogen (Giroud et al., 2000; Sheffler and Roth, 2003). Studies in the 1980s identified an active component of *S. divinorum* to be salvinorin A (Ortega et al., 1982; Valdes et al., 1983), which was later determined to be the primary psychoactive molecule in the plant (Siebert, 1994). Salvinorin A is a neoclerodane diterpene whose absolute configuration and structure has been determined by NMR and single-crystal x-ray analysis (Ortega et al., 1982; Koreeda et al., 1990; Valdes, 1994) and is quite distinct from other natural hallucinogens. The putative molecular target of salvinorin A remained elusive until recently, when radioligand, GTPγS and adenyl cyclase assays all identified salvinorin A to be an agonist at κ-opioid receptors (KORs) *in vitro* (Roth et al., 2002; Chavkin et al., 2004; Nichols, 2004; Yan and Roth, 2004). Importantly, there is no *in vitro* agonist activity at the 5-HT_{2A} serotonin receptors that mediate actions of most other known hallucinogens (Roth et al., 2002; Chavkin et al., 2004; Nichols, 2004).

These earlier studies established that salvinorin A was a potent and selective KOR agonist. However, it is still unclear whether salvinorin A is selective for κ_1 or κ_2 opioid receptors. The κ_2 opioid receptor was initially defined on the basis of ligand binding and physiological studies (Iyengar et al., 1986; Zukin et al., 1988; Rothman et al., 1990). Although there is no evidence for a gene coding for the κ_2 opioid receptor, recent data suggests that the κ_2 opioid receptor might result from δ–κ heterodimers (Jordan and

Devi, 1999). We have used ligand binding to determine whether salvinorin A shows selectivity for κ_1 or κ_2 opioid receptors.

Consistent with *in vitro* data, the reported physiologic effects of salvinorin A are consistent with prospective action through the KOR. For example, documented *in vivo* effects of *S. divinorum* and purified salvinorin A include sedation, antinociception, and production of hallucinations (Siebert, 1994; Valdes, 1994; Giroud et al., 2000; Hanes, 2001; Wang et al., 2005; McCurdy et al., 2006). Sedation and antinociception have long been identified as major effects of KOR agonists (Martin et al., 1976; Vonvoigtlander et al., 1983; Leighton et al., 1988) while activation of KORs has also been noted to be dysphoric and to cause perception alterations (Pfeiffer et al., 1986). Consistent with the dysphoric effects in humans, administration of salvinorin A to mice produces a long-lasting decrease in extracellular dopamine (Zhang et al., 2005) that is consistent with KOR activation (Chefer et al., 2005). Based on the overall similarities between the behavioral effects of salvinorin A and κ agonists, we hypothesized that KOR could be the target for many if not all salvinorin A actions *in vivo* and thus could potentially mediate additional behavioral responses, such as hypothermia (Baker and Meert, 2002), that result from KOR-1 activation. The most direct way to test the *in vivo* requirement of KOR-1 is in a knockout (KO) model, so we have compared the responses of several salvinorin-related compounds in both wild-type mice and in a novel mouse strain containing a null mutation of the KOR-1 gene.

Methods

All experiments were conducted in accordance with the guidelines of the Institutional Care and Use Committees of UMDNJ-RWJMS and the National Institute on Drug Abuse (NIDA), National Institutes of Health in facilities accredited by the American Association for the Accreditation of Laboratory Animal Care (AAALAC). Adult male mice were used for all initial characterization of the KOR-1 KO mice and were derived from mating of either KOR-1 heterozygous mutant mice maintained on a mixed C57Bl6/J x 129S6 background or wild-type and KOR-1 mutant mice maintained on an inbred 129S6 background. Subsequent behavioral experiments used wild-type and KOR-1 KO mice of both sexes maintained on the 129S6 background.

Production of KOR-1 deficient mice.

A digoxygenin-labeled cDNA probe containing murine KOR-1 exons 2 and 3 (Yasuda et al., 1993) was used to screen a genomic library constructed from the 129/SwRe strain. One genomic clone containing exon 3 of KOR-1 was isolated, confirmed by sequencing, and used to construct the targeting vector. A 6 kb Not I/Spe I fragment 5' of exon 3 and a 1.4 kb EcoRI fragment downstream of exon 3 were subcloned into pBS-KO vector (obtained from Dr. Steven Potter, U. Cincinnati) containing neomycin (neo) and thymidine kinase selection markers. Replacement of exon 3 with a neo cassette would then occur following successful homologous recombination (Fig. 1A).

The targeting vector was linearized and electroporated into 129SvEv-derived CCE ES cells (provided by Elizabeth Robertson) as described (Schuller et al., 1999). Individual ES colonies that survived the G418 and gancyclovir double selection were

screened, and targeted ES cell lines were identified following hybridization with a 0.6 kb screening probe from a genomic region 3' of the region incorporated into the targeting vector. This probe hybridized to a 8kb wild-type fragment and a diagnostic 5kb band derived from the targeted allele following BamHI digestion (Fig. 1B). Targeted ES cells were injected into blastocysts and chimeras were mated with C57Bl6/J and 129S6 female mice. Heterozygous mice were identified using the screening strategy described above and used to establish and maintain the mixed C57BL/6 x 129S6 strain as well as produce an inbred 129S6 line.

Homogenate binding assays.

Adult brains from wild-type and homozygous KOR-1 mutant mice were isolated and homogenized in 30 volume of 50 mM Tris HCl, pH 7.4 at 4˚C, and then centrifuged twice at 30,000xg for 15 minutes at 4˚C with the supernatant discarded. Brain membranes were suspended in 30 volume of buffer and incubated for 30 minutes at 37˚C to dissociate bound endogenous ligand before recentrifugation.

Resuspended brain aliquots (400 mg wet weight in 30 ml Tris buffer, 400 µl aliquots of membrane suspension (~0.25 mg protein) were incubated with eight concentrations of [^{3}H]-U69,593 (Dupont, NEN) in 50 mM Tris HCl, pH 7.4 for 90 minutes. Non-specific binding was assessed using 10 nM naloxone. The homogenates were filtered under reduced pressure through Whatman GF/B filters using a Brandel cell harvester. Filters were washed three times with 50 mM Tris HCl, pH 7.4, at 4˚C to remove free radioligands and then assayed by liquid scintillation spectrometry. Protein concentrations were determined by the Lowry procedure (Sigma). Binding affinities and capacities were determined by Scatchard analysis (Munson, 1983).

Chemistry.

Salvinorin A was isolated from dried leaves of *S. divinorum* by the method reported by Valdes et al. (1994). Salvinorin B and salvinorinyl-2-propionate were prepared as previously detailed (Chavkin et al., 2004). Salvinorin B was characterized by ^{1}H-NMR, ^{13}C-NMR, and high-resolution mass spectrometry (HRMS) and found to be authentic by comparison with literature values (Valdes, 1994). The reported esters were characterized by HPLC and HRMS. NMR (^{1}H and ^{13}C) spectra were recorded on a Bruker AMX-NMR spectrometer in $CDCl_3$. The HRMS spectra were measured using a Bioapex FT mass spectrometer with electrospray ionization. HPLC was conducted on a Waters Deltaprep 4000 system using a Waters Xterra RP_{18}, 5 μm, 4.6 mm x 150 mm column, with mobile phase H_2O/Acetonitrile (1:1). TLC analyses were carried out on pre-coated Si gel G_{254}, 250 μm plates, with the developing system hexane/EtOAc (2:1) and visualized with vanillin/H_2SO_4 in EtOH.

Radioligand binding assays.

Assays of rat brain κ_2 opioid receptor binding sites followed published procedures (Rothman et al., 1992). Briefly, membranes prepared from whole rat brain, pretreated with 1 μM 2-(p-ethoxybenzyl)-1-diethylaminoethyl-5-isothiocyanatobenzimidazole-HCL (BIT) and 1 μM N-phenyl-N-[1-(2-(p-isothiocyanato)phenylethyl)-4-piperidinyl]propanamide-HCL (FIT) to deplete μ and δ opioid receptor binding sites, washed by repeated centrifugation and resuspension, and then assayed with [^{3}H]bremazocine (2.5 nM) in 50 μM potassium phosphate buffer, pH 7.4, for 4-6 hr at 0°C. The incubations were terminated by rapid filtration over Whatman GF/C filters. Further details of this assay are published (Rothman et al., 1990).

Drug administration

All experimental drugs were dissolved in 50%, 60% or 100% DMSO. Controls contained equal concentrations of DMSO mixed with saline. Intracerebroventricular (ICV) injections were performed as described elsewhere (Haley, 1957). Briefly, mice were exposed to an isoflurane and oxygen combination for approximately 2 to 3 min until full anesthesia was observed. A midline incision was made along the sagittal suture and a microinjection of 3 μl was administered in the lateral ventricle at 2 mm anterior to the lambda suture and 3 to 3.5 mm lateral to the midline suture that extended 2 mm below the surface of the skull. Nearly complete locomotor/nociceptive recovery from anesthesia was observed within 15 minutes.

Analgesic testing

Analysis of salvinorin A, salvinorin B and salvinorinyl-2-propionate analgesia was performed on wild-type and KOR-1 KO mice of both sexes maintained on a 129S6 inbred background using the radiant heat tail flick assay of nociception. Intensity of the beam was adjusted to yield baseline tail flick latencies between 2-3 seconds and cut off of 10 seconds was employed to reduce tissue damage. Percent maximum possible effect (%MPE) was determined according to the following formula: [(post-injection latency-pre-injection latency)/(10-pre-injection latency)] x 100. Nociceptive thresholds were determined prior to drug administration. Mice were then injected ICV with drug and tested for analgesia 15 minutes afterward. For time courses, mice were injected ICV with drug and tested for analgesia every 15 min for 1 hour. For cumulative dose response curves, mice were injected ICV with drug and tested for analgesia 15 minutes afterward. Immediately after testing mice were injected with the next highest dose. This procedure

was repeated until all doses had been administered. Initial experiments were analyzed using one-way ANOVA. Differences were isolated using Fisher's PLSD. Time-dependence was evaluated using repeated measures ANOVA with time as the dependent variable and treatment as an independent variable. Individual differences were isolated using one-way ANOVA at individual time points and Fisher's PLSD for post-hoc tests. Dose-dependent analgesia was evaluated using repeated measures ANOVA with dose as the dependent variable ($p<0.05$). Both salvinorin A and salvinorinyl-2-propionate show a significant effect of dose in wild type mice. Salvinorin B in wild type mice and salvinorin A and salvinorinyl-2-propionate in KOR-1 KO mice do not show a significant effect of dose. Individual doses were analyzed using Fisher's PLSD to determine significance between wild type and KOR-1 KO responses. ED_{50} values were determined using a non-linear curve-fitting program for data expressed as both percent MPE and percent mice that became analgesic (Graph-Pad Prizm, Graph Pad Software, San Diego, CA). Mice were considered analgesic when post injection latency was greater than twice baseline line tail flick latency. No difference in drug response was observed between sexes.

Rectal temperature measurement

Effects of salvinorin A and salvinorinyl-2-propionate on rectal body temperature was performed on wild-type and KOR-1 KO mice of both sexes maintained on a 129S6 inbred background. All measurements were made with a 2.5 cm rectal temperature probe (Model BAT-12, Physitemp, Clifton, NJ). The probe was inserted 2.5 cm, allowed to reach temperature for 5-6 seconds, and the measurement then recorded. Rectal temperature was first determined prior to drug administration. Mice were then injected ICV with drug and then measured for rectal body temperature every 15 minutes for 2

hours. Differences were isolated using one-way ANOVA at individual time points with treatment as the independent factor and Fisher's PLSD for post-hoc tests. No difference in drug response was observed between sexes.

Results

Pharmacological characterization of salvinorin A, salvinorin B and substituted salvinorin A derivatives.

Both salvinorin A and salvinorinyl-2-propionate significantly inhibit κ_1 opioid receptor binding, while neither salvinorin A nor salvinorinyl-2-propionate significantly inhibited κ_2 opioid receptor binding (Table 1). In contrast to the weak interaction of salvinorin A with κ_2 opioid receptors in rat brain, other agents that activate pharmacologically defined κ_2 opioid receptors, such as (-)-ethylketocyclazocine (Sheffler and Roth, 2003), have high affinity for the κ_2 opioid receptor site (Iyengar et al., 1986). Thus, based on receptor binding affinity salvinorin A and its derivatives are κ_1 opioid receptor selective.

Production and pharmacologic characterization of KOR-1 deficient mice.

To evaluate the actions of salvinorin related compounds *in vivo*, a novel line of KOR-1 KO mice was utilized. ES cells from one of two KOR-1 targeted ES clones were injected into blastocysts and germ-line transmitting chimeras were identified. Mice heterozygous for the mutant KOR-1 allele were mated and homozygous mutant mice were identified that were viable and fertile with no obvious morphological abnormalities (Fig. 1C). Genotypes of offspring from heterozygous matings arose in the predicted Mendelian frequency (wild-type 28.2%, heterozygous 48.4%, and homozygous 23.4%; n=273). The effect of the exon 3 KOR-1 gene deletion on binding of the selective κ agonist [^{3}H]U69,593 was determined by homogenate binding assays on adult brain membrane fractions from +/+, +/-, and -/- genotypes. U69,593 binding was absent from KOR-1 KO mice, while binding in heterozygous mice was ~50% that of wild-type levels

(Fig. 1D) similar to another line of KOR-1 KO (Simonin et al., 1998) mice in which binding CI-977 binding was measured.

Antinociceptive effects of salvinorin A, salvinorin B and salvinorinyl-2-propionate

To determine if salvinorin A has analgesic potency, we initially injected salvinorin A at a dose of 5 mg/kg IP and observed no effect 30 minutes later (data not shown; see also Wang et al, 2005). We next determined if salvinorin A has potency when injected supraspinally. 7.5 μg of salvinorin A injected ICV shows antinociceptive potency when nociception is measured 15 minutes later (Fig. 2A). We next tested salvinorinyl-2-propionate and salvinorin B supraspinally at the same dose (Fig. 2A). Consistent with the slight reduction in affinity compared to salvinorin A observed in previous studies (Chavkin et al., 2004), salvinorinyl-2-propionate produced a more limited antinociception at 7.5 μg than salvinorin A. In contrast, salvinorin B demonstrated no analgesic potency. We then increased the dose to 13 μg for savinorinyl-2-propionate and salvinorin B (Fig. 2B) and measured nociception 15 minutes later. At this dose, salvinorinyl-2-propionate produced a significant antinociceptive response while salvinorin B remained inactive. Thus *in vivo* analgesic activity could be elicited by only salvinorin A-like compounds active *in vitro*.

To further characterize salvinorin A-elicited analgesia, time course studies for the analgesic actions of salvinorin A and salvinorinyl-2-propionate were performed. ICV injection of either 7.5 μg salvinorin A or 10 μg salvinorinyl-2-propionate produced significant analgesia as early as 15 minutes (Fig. 3). By 30 minutes the tail flick latencies for both the salvinorin A and salvinorinyl-2-propionate-injected groups were still elevated but not significantly different than controls. By 45 minutes after injection, the

tail-flick latencies for all treated mice had returned to baseline values. To better compare drug time-courses between behavioral paradigms (e.g. hypothermic effects, see Fig.6 below), antinociceptive effects were also evaluated following ICV injection of 50 μg salvinorin A. As seen in figure 3, injection of this supermaximal dose of salvinorin A increased the time-course for significant drug action. Salvinorin A still acts rapidly and reaches 100% MPE within 15 minutes, but a significant drug effect is maintained for at least 45 minutes.

Dose-response curves examining the potency of salvinorin A, salvinorinyl-2-propionate and salvinorin B analgesia were then produced with nociception measured 15 minutes post-injection. Both salvinorin A and salvinorinyl-2-propionate induced dose-dependent antinociception in wild-type mice with an ED_{50}s of 1.5 μg and 2.0 μg (for data expressed as % MPE) or 1.4 μg and 1.7 μg (for data expressed as % mice analgesic) respectively (Fig. 4A, 4B), while salvinorin B was inactive in wild-type mice (Fig. 4C). As further demonstration that salvinorin A and salvinorinyl-2-propionate act specifically on KORs, neither salvinorin A nor salvinorinyl-2-propionate induced analgesia in KOR-1 KO mice (Fig. 4A, 4B).

Because ICV injection of salvinorin A and salvinorinyl-2-propionate in KOR-1 KO mice and salvinorin B in wild-type mice increased %MPE slightly at the highest doses of the dose-response curve, we wanted to distinguish whether this effect was an antinociceptive response at high doses of the compound or a nonspecific antinociceptive effect of the vehicle (100% DMSO) in which test compounds were dissolved. ICV injection of 100% DMSO alone consistently stimulated a greater antinociceptive response (~20%; Fig. 2) than previously seen following ICV injection of saline alone (~10%; data

not shown). We then assessed whether repeated injections of 100% DMSO ICV produced significant antinociception above this baseline value. Fig. 4D shows that repeated DMSO injection does induce slight antinociception in wild-type mice following repeated dosing compared to saline injection. However, the level of analgesia observed is significantly lower than that observed following ICV injection of salvinorin A or salvinorinyl-2-propionate in wild-type mice and never reached statistical significance (Fig. 5). Thus, the level of nociception in dose-response curves for salvinorin B in wild-type mice or salvinorin A or salvinorinyl-2-propionate in KOR-1 KO mice following the final ICV injection is comparable to the level of nociception observed following repeated ICV injection of vehicle (100% DMSO) (Fig. 5).

Effects on rectal body temperature

Because KOR agonists are known to cause depression of rectal body temperature, we attempted to ascertain whether salvinorin A and salvinorinyl-2-propionate also affect rectal body temperature. Rectal body temperature is immediately suppressed in both control and drug-treated wild-type mice following ICV injection, because of anesthesia used during the procedure (Kushikata et al., 2005). However, rectal body temperature of vehicle-treated mice returns to baseline within 45-60 minutes of injection whereas rectal body temperature of mice treated with 50 μg of salvinorin A or salvinorinyl-2-propionate shows prolonged suppression at least 120 minutes post-ICV injection (Fig. 6A, 6B). Although a trend for hypothermia following ICV injection of 7.5 μg of salvinorin A is seen at 45 minutes post-injection, no significant effect on rectal body temperature was observed in wild type mice (Fig 6A). ICV injection of salvinorin B does not produce additional suppression of rectal body temperature compared to vehicle-injection (Fig.

6C). Finally, to confirm that the reduction in rectal body temperature induced by ICV injection of salvinorin A requires the KOR-1 gene, we injected salvinorin A ICV in KOR-1 KO mice. Similar to the ICV injection of salvinorin B, no difference was seen in rectal body temperature between vehicle and salvinorin A injected KOR-1 KO mice (Fig. 6D).

Discussion

The major finding of the present study is the genetic determination that the *in vivo* actions of salvinorin A and its propionate-derivative are KOR-1 dependent. Both salvinorin A and its derivative salvinorinyl-2-propionate have antinociceptive and hypothermic effects when injected ICV into wild-type mice (Fig. 2A, 2B; Fig. 6A, 6B), while both drugs were inactive in KOR-1 KO mice (Fig. 4A, 4B; Figure 6D), demonstrating that a principal molecular target of these compounds *in vivo* is the KOR. The loss of antinociceptive and hypothermic effects of salvinorin A in the KOR-1 KO mouse does not rule out the possibility that salvinorin A has other sites of action for other behavioral responses still to be investigated, but what these other sites of action might be is unclear since prior studies have failed to find any other molecular target for the actions of salvinorin A, despite screening a large number of neurotransmitter receptors, ion channels and transporters (Roth et al., 2002). Taken together, the most parsimonious explanation is that the effects of salvinorin A *in vivo* are mediated principally, if not exclusively, by KORs. Indeed, recent reports have suggested that the psychological effects of salvinorin A in humans (Sheffler and Roth, 2003) and discriminative stimulus effects of salvinorin A in monkeys (Butelman et al., 2004) are mediated by KORs.

Consistent with recent studies (Wang et al., 2005; McCurdy et al., 2006) showing that salvinorin A has low potency and a very short half-life *in vivo*, we demonstrate antinoceptive efficacy only for a transient time following an ICV injection of an ED80 dose (7.5 μg) of salvinorin A (maximal response at 15 minutes and no effect by 45 minutes; Figure 3). Hypothermic effects of salvinorin A are absent following ICV

injection of the same 7.5 μg dose of salvinorin A (Figure 6A). It is possible that salvinorin A may be efficacious at 7.5 μg ICV, but the hypothermic effects of the anesthesia required for the ICV surgery may mask these effects at early time points. ICV injection of 25 μg salvinorin A was also inactive in producing hypothermia (data not shown). We obtained significant hypothermic effects only following ICV injection of 50 μg Salvinorin A (Figure 6A). The hypothermic efficacy of salvinorin A at this higher dose appears to be relatively longer lasting (maximal effect after 75 minutes and significant effects out to 120 minutes) than significant antinociceptive effects. The remaining difference in time-course may be due to physiological differences between the two behaviors. Although not well documented, differences in maximal time for kappa opioid agonist action on nociception and rectal temperature have previously been observed. For example, Spencer et al. demonstrated that although U50,488H administered ICV to rats shows maximal antinociceptive efficacy after 10 minutes and all analgesic action is lost after 40 minutes, the same treatment has maximal efficacy on rectal temperature after 20 minutes with significant effects still present 40 minutes later (Spencer et al., 1988). Similar differences in time course are seen when comparing maximal efficacy for dynorphin A (1-13) administered ICV. Nakazawa et al. showed a maximal efficacy for ICV administered dynorphin A at 15 minutes post injection (Nakazawa et al., 1985), whereas dynorphin A (1-13) effects on rectal body temperature reach maximal effect 60 minutes post injection and are still present 2 hours after the initial injection (Chen et al., 2005). Exactly why such a difference exists between the time course of effects of kappa agonists on nociception and rectal temperature is not known. However, the difference may be due to how activation of kappa opioid receptors

influences these behaviors as well as the time required for physiological changes in these behaviors to be manifested. Kappa opioid activation may rapidly affect nociception by directly inhibiting the nociceptive neural circuitry. Following degradation of the drug, the effects on nociception disappear as rapidly as they appear. Changes in rectal body temperature may take longer to appear following kappa opioid activation due to the requirement for changes in physiology to lower body temperature and may last longer due to the same requirement for physiological alterations to restore the body temperature following drug clearance.

Finally, although the vehicle, DMSO, used in these studies has slight behavioral activity, it does not confound interpretation of the data. High concentrations of DMSO (50% or greater) were used to dissolve salvinorin A because of its relative insolubility in common vehicles used for ICV injection. After a single ICV injection of DMSO we saw ~ 20% MPE. Subsequent ICV injections revealed that this effect increased to a maximum of ~45% following a fourth injection (Figure 4D). These results are consistent with literature showing that DMSO can have antinociceptive actions (Haigler and Spring, 1981; Haigler and Spring, 1983). Testing salvinorin A in both wild type and KOR-1 KO mice eliminates any ambiguity in our interpretation of these data. The decrease in the antinociceptive effect of salvinorin A seen in the KOR-1 KO could only be due to the loss of salvinorin A action on the kappa opioid receptor. The remaining antinociceptive effect could then be attributed to DMSO. Therefore, by testing salvinorin A in wild type and KOR-1 KO mice we can definitively state that some if not all of salvinorin A antinociceptive effects are due to action at the kappa opioid receptor. In addition, the slight elevation of % MPE in the dose response curve from wild-type mice injected with

salvinorin B is most likely not due to antinociceptive activity of the compound, but rather action of DMSO vehicle (Figures 5).

In conclusion these studies accomplished several objectives. First, we have confirmed two behavioral responses for salvinorin A and one of its derivatives. These compounds have analgesic efficacy and depress rectal body temperature consistent with their identification as KOR agonists. Second, we show that salvinorin B is inactive at comparable doses indicating that salvinorin A is, most likely, the main active component of *S. divinorum in vivo*, as it is *in vitro*. In addition we provide the first *in vivo* structure-function studies of salvinorin A at the KOR and have identified salvinorinyl-2-propionate as a novel salvinorin A derivative with appreciable KOR actions *in vivo*. We also demonstrate that salvinorin A interacts with the κ_1 opioid receptor subtype, but not the κ_2 opioid receptor subtype. Finally, by performing behavioral assays in wild-type and a novel strain of KOR-1 KO mice, we genetically confirm that one *in vivo* site of action for salvinorin A is the KOR and thus demonstrate that salvinorin A is a functional KOR agonist with behavioral consequences when injected ICV.

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Footnotes

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Legends for Figures

Figure 1. Targeting of KOR-1. (A) KOR-1 genomic clone #3 was restriction mapped and exon 3 was positioned ~8 kb from the 5' end and ~4 kb from the 3' end of the clone. The KOR-1 targeting vector was constructed by subcloning ~6 kb 5' of exon 3 and 1.4 kb 3' of exon 3 into the KO vector such that the neo gene replaced a 2 kb KOR-1 sequence containing exon 3. In the predicted targeted locus, the neo gene would introduce an extra Bam HI site. Using a screening probe outside the sequence used for targeting, Southern blot analysis detects an 8kb wild-type allele and a 5kb targeted allele following homologous recombination. This screening strategy was used to identify a positively targeted ES cell line after electroporation (B) and to demonstrate mice of all genotypes from offspring of heterozygous mating (C). (D) [^{3}H]U69, 593 was used to assess kappa receptor binding in adult brain homogenates from wild-type, heterozygous, and homozygous KOR-1 littermates. Binding was undetectable in KOR-1 mice and reduced to ~50% in heterozygous mice. (+/+: Bmax=8.2 fmol/mg protein; Kd= 0.64 nmol; +/-: Bmax=3.24 fmol/mg protein; Kd=0.59; -/-: Bmax=N/A; Kd= N/A (n=3).

Figure 2. Salvinorin A and salvinorinyl-2-propionate elicit antinociception. (A)Wild-type mice were injected ICV with 7.5 μg salvinorin A (black bars), salvinorinyl-2-propionate (dotted bars) salvinorin B (diagonal stripped bars) dissolved in 50% DMSO or 50% DMSO (Control) (white bars) n=6-18; *=$p<0.05$ vs. Control. (B) Wild-type mice were injected ICV with 13 μg of salvinorinyl-2-propionate (dotted bars), salvinorin B (diagonal striped bars) dissolved in 100% DMSO or 100% DMSO (control) (white bars) n=4-9; *=$p<0.05$ vs. Control.

Figure 3. Salvinorin A and salvinorinyl-2-propionate antinociception is short acting. Wild-type mice were injected ICV with 3 μl 100% DMSO (control) (open triangles), 7.5 μg salvinorin A (closed squares), 50 μg salvinorin A (open squares), or 10 μg salvinorinyl-2-propionate (closed circles) dissolved in 100% DMSO. Data is graphed as mean ± SEM. n=6-8. *=$p<0.05$ (both 7.5 and 50 μg) salvinorin A vs. Control; salvinorinyl-2-propionate vs. Control.

Figure 4. Salvinorin A and salvinorinyl-2-propionate produced antinociception through activation of the KOR. (A) Wild-type (closed squares) or KOR-1 KO (open squares) mice were injected ICV with an escalating dose of salvinorin A (1.5-15 μg) dissolved in 100% DMSO. Data is graphed as mean ± SEM. n=9 for wild-type; n=8 for KOR-1 KO. *=$p<0.05$ Wild-type vs. KOR-1 KO. (B) Wild-type (closed squares) or KOR-1 KO (open squares) were injected ICV with an escalating dose of salvinorinyl-2-propionate (1-30 μg) dissolved in 100% DMSO. Data is graphed as mean ± SEM. n=8 for wild-type; n=6 for KOR-1 KO. *=$p<0.05$ Wild-type vs. KOR-1 KO. (C) Wild-type mice (closed squares) were injected ICV with an escalating dose of salvinorin B (1-30 μg) dissolved in 100% DMSO. Data is graphed as mean ± SEM. n=9. (D) Wild-type mice were repeatedly injected ICV with either saline (closed circles) or 100% DMSO (open triangles). Data is graphed as mean ± SEM. n=5 for saline; n=7 for DMSO.

Figure 5. Repeated ICV injection of DMSO slightly elevates % MPE. Mice were repeatedly injected four times ICV with salvinorin A, (n=9 for wild-type (open bars); n=8 for KOR-1 KO (closed bars)); salvinorinyl-2-propionate (n=8 for wild-type; n=6 for KOR-1 KO) or salvinorin B (n=9) dissolved in 100% DMSO, 100% DMSO (n=9) or

saline (n=5). Data presented is the final drug and/or vehicle injection value from figures 4A-D. Data is graphed as mean ± SEM. *=p<0.05 vs. wild-type 100% DMSO injected.

Figure 6. Salvinorin A and salvinorinyl-2-propionate reduce rectal body temperature via the κ opioid receptor. (A) Wild-type mice injected ICV with 100 % DMSO (control) (closed circles) 7.5 μg salvinorin A (closed squares), or 50 μg salvinorin A (open squares) dissolved in 100% DMSO. Data is graphed as mean ± SEM. n=4-7.*=p<0.05 salvinorin A vs. Control. (B) Wild-type mice injected ICV with 100 % DMSO (Control) (closed circles) or 50 μg salvinorinyl-2-propionate (closed squares) dissolved in 100% DMSO. Data is graphed as mean ± SEM. n=7. *=p<0.05 salvinorinyl-2-propionate vs. Control. (C) Wild-type mice injected ICV with 100 % DMSO (Control) (closed circles) or 50 μg salvinorin B (closed squares) dissolved in 100% DMSO. n=5. (D) KOR-1 KO mice injected ICV with 100% DMSO (Control) (closed circles) or 50 μg salvinorin A (closed squares) dissolved in 100% DMSO. Data is graphed as mean ± SEM. n=6.

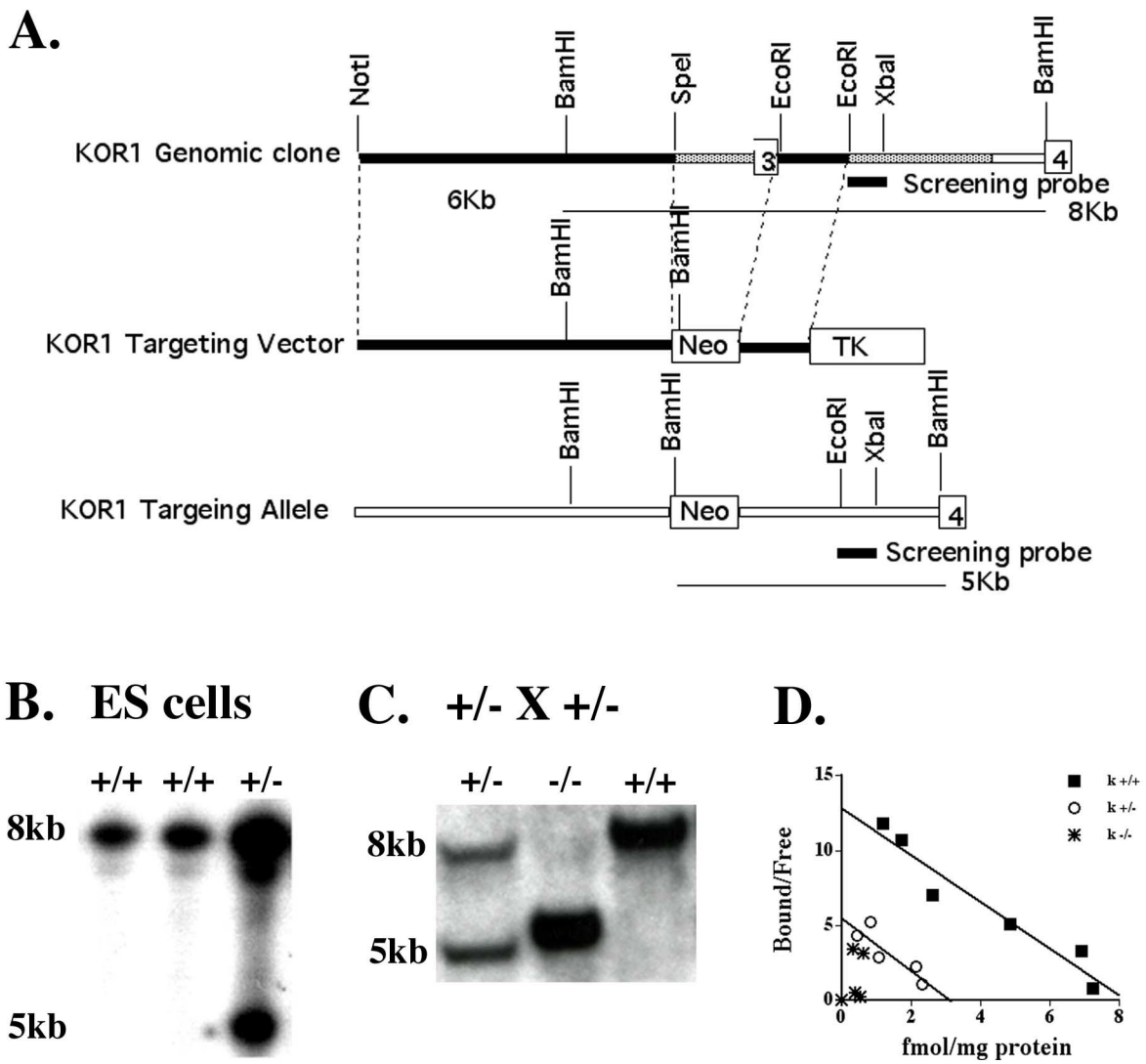
Tables

Table 1. Effect of Salvinorin A derivatives on KOR Subtype Binding and KOR-1 Stimulated Ca^{++} Mobilization

Drug	Ki at KOR-1 (nM±SEM)	Ki at KOR-2A (nM±SD)	Ki at KOR-2B (nM±SD)	pEC50 (nM±SD)[1]	Emax[1]
U69,593	0.7±0.05[2]	121±10[3]	>10,000[3]	1.14±0.02	100
U50,488H	0.2±0.05[2]	55.6±7.4[3]	20,400±5160[3]	1.39±0.14	102±4
Salvinorin A	18.7±3.4[1]	>10,000	>10,000	0.84±0.07	104±7
Salvinorinyl-2-propionate	32.6±15.7[1]	ND	ND	1.23±0.18	102±8
Salvinorin B	>10,000[1]	>10,000	>10,000	NA	NA

KOR-2 binding experiments were done as described in Methods. [1]The results for KOR-1 binding and KOR-1 stimulated Ca^{++} mobilization are taken from (Chavkin et al., 2004). [2]Data from (Toll et al., 1998). [3]Data from (Rothman et al., 1990). The salvinorin A derivatives were also screened using a large panel of cloned human receptors, ion channels and transporters (see (Roth et al., 2002) for list) and were inactive (data not shown). NA=no activity. ND=not done.

Figure 1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EcoRI
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KOR1 Genomic clone
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BamHI
BamHI
KOR1 Targeting Vector
Neo
TK
BamHI
BamHI
EcoRI
XbaI
BamHI
KOR1 Targeing Allele
Neo
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Screening probe
5Kb
B. ES cells
+/+ +/+ +/-
8kb
5kb
C. +/- X +/-
+/- -/- +/+
8kb
5kb
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k -/-
Bound/Free
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5
0
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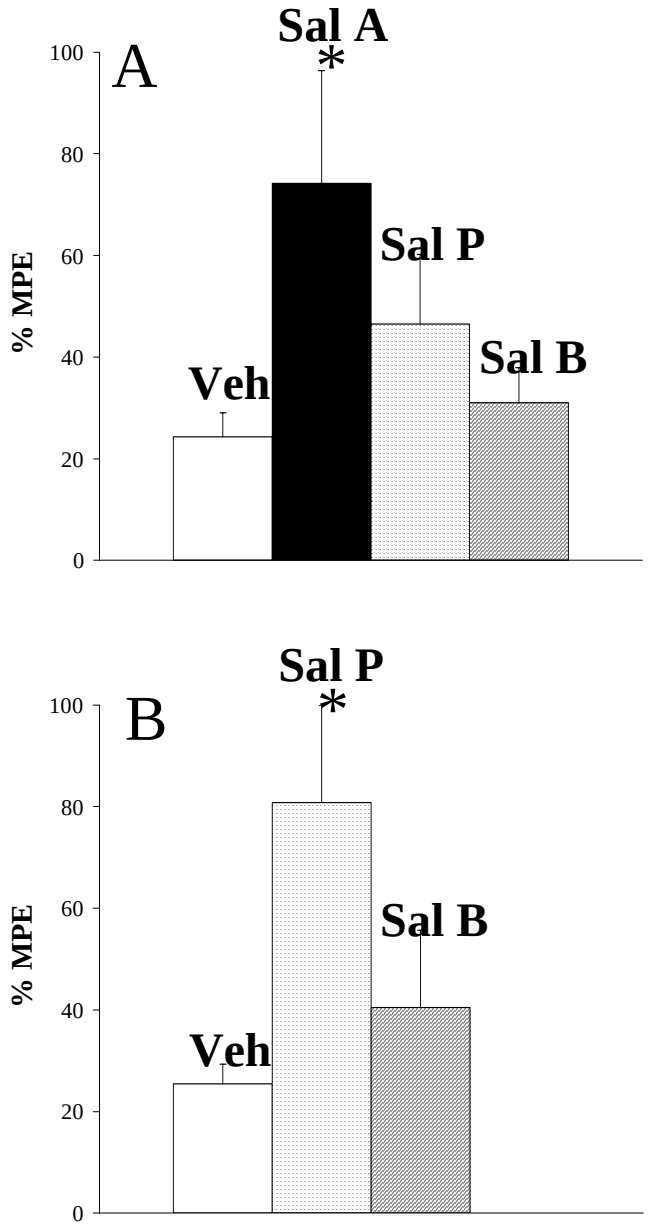
2
4
6
8

Figure 2


A
Sal A
*
Sal P
Sal B
Veh
% MPE
100
80
60
40
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B
Sal P
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Sal B
Veh
% MPE
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40
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Figure 3

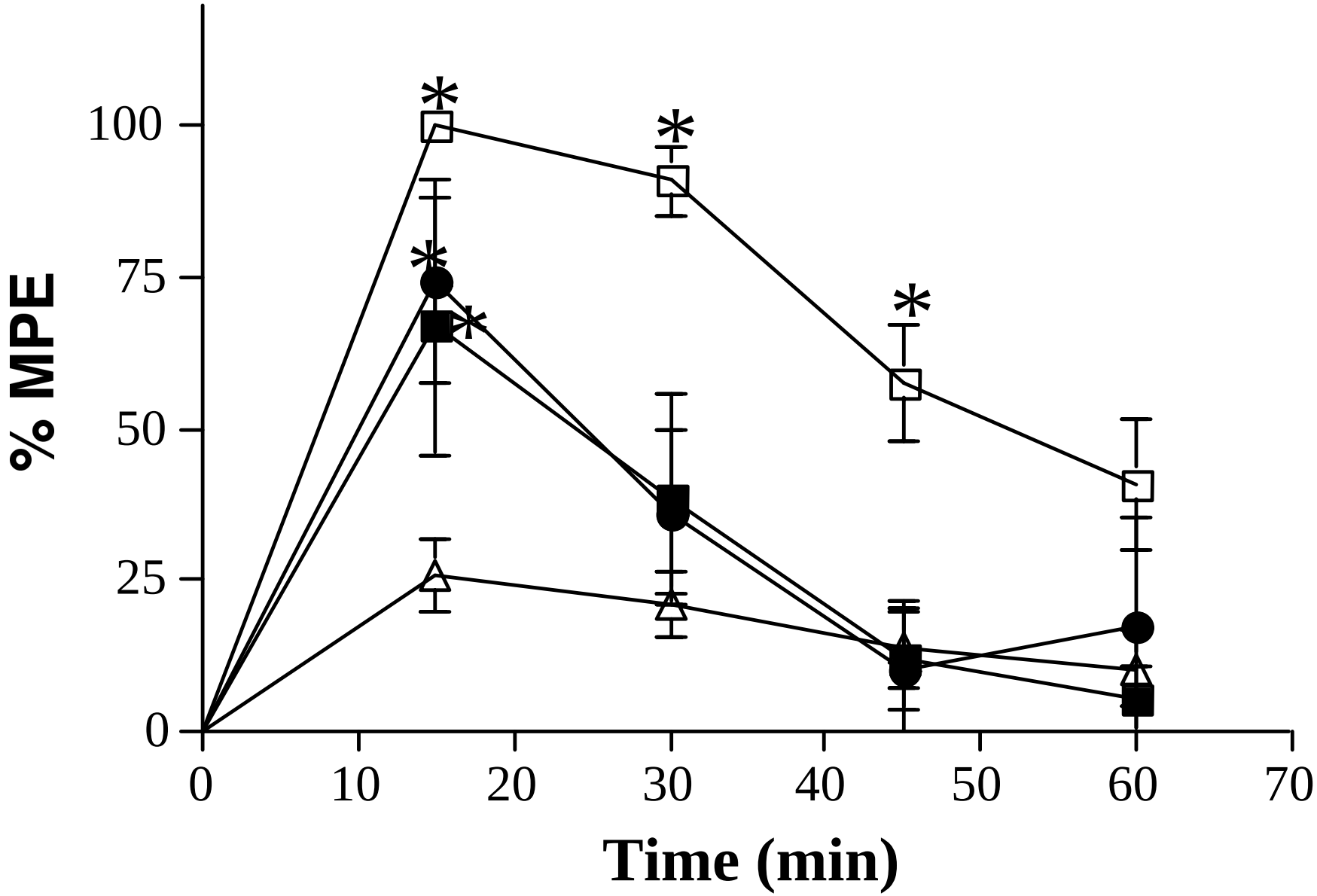
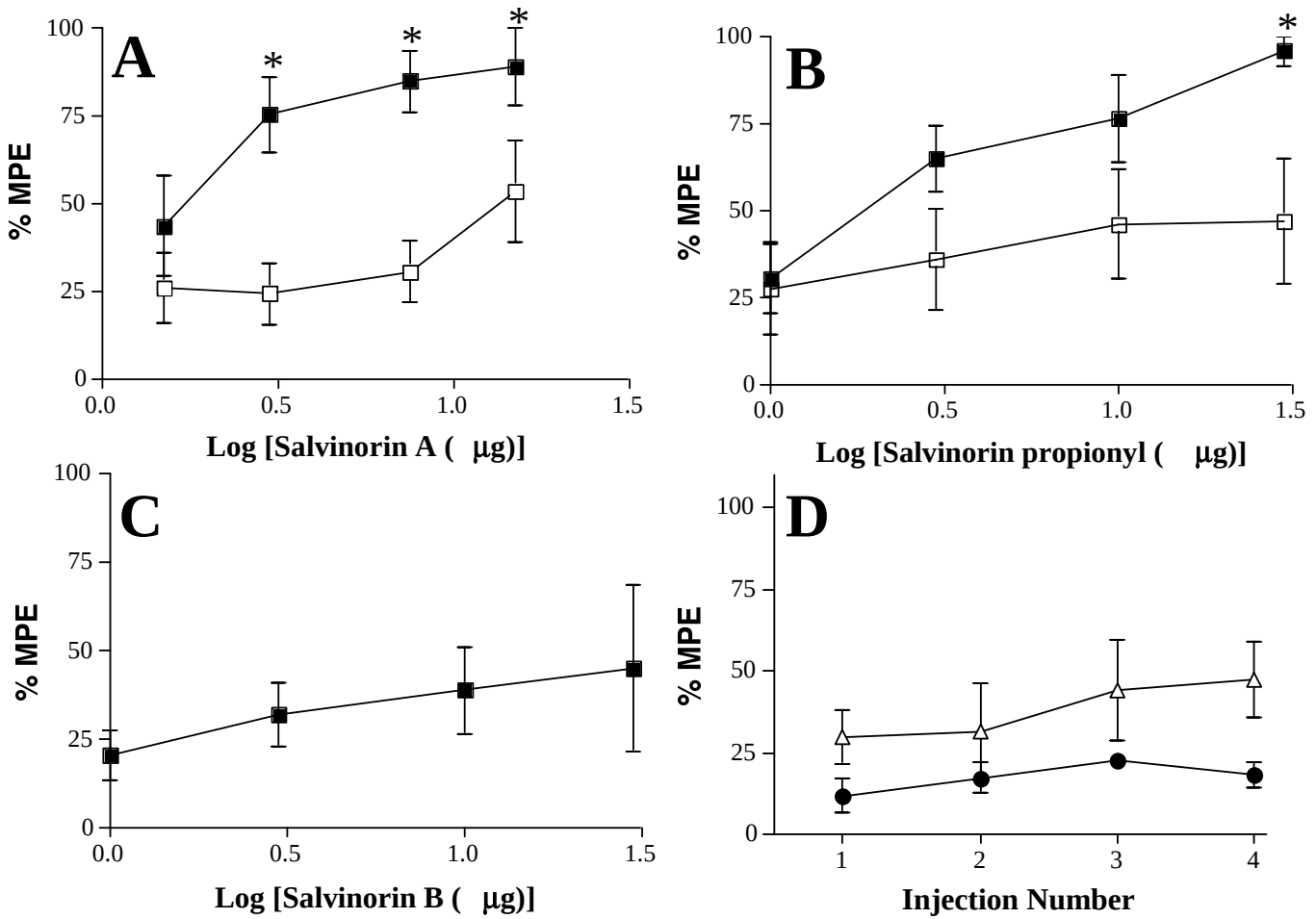


Figure 4


A
B
C
D
% MPE
Log [Salvinorin A (μg)]
Log [Salvinorin propionyl (μg)]
Log [Salvinorin B (μg)]
Injection Number
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50
75
100
0.0
0.5
1.0
1.5
1
2
3
4
*

Figure 5

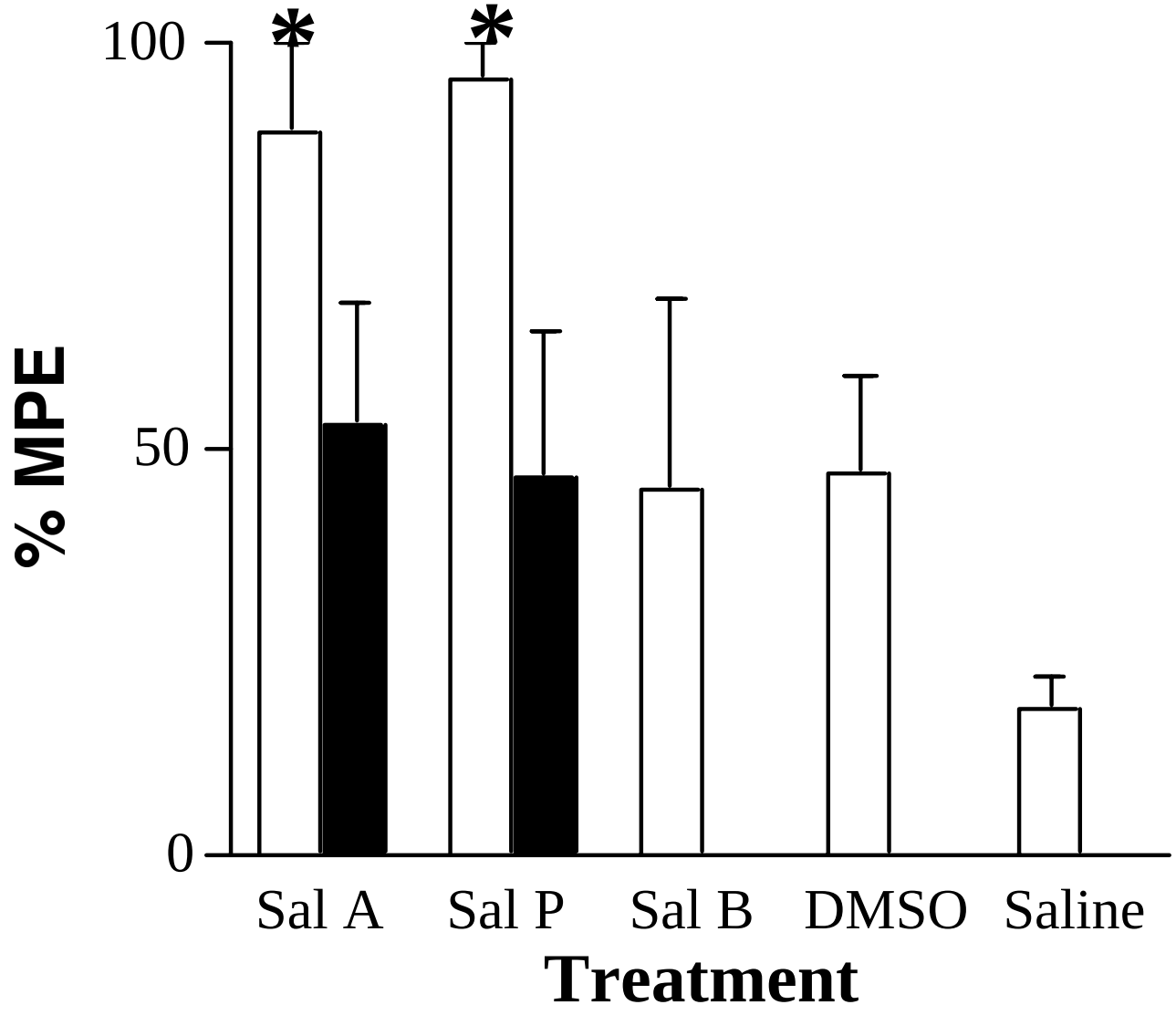
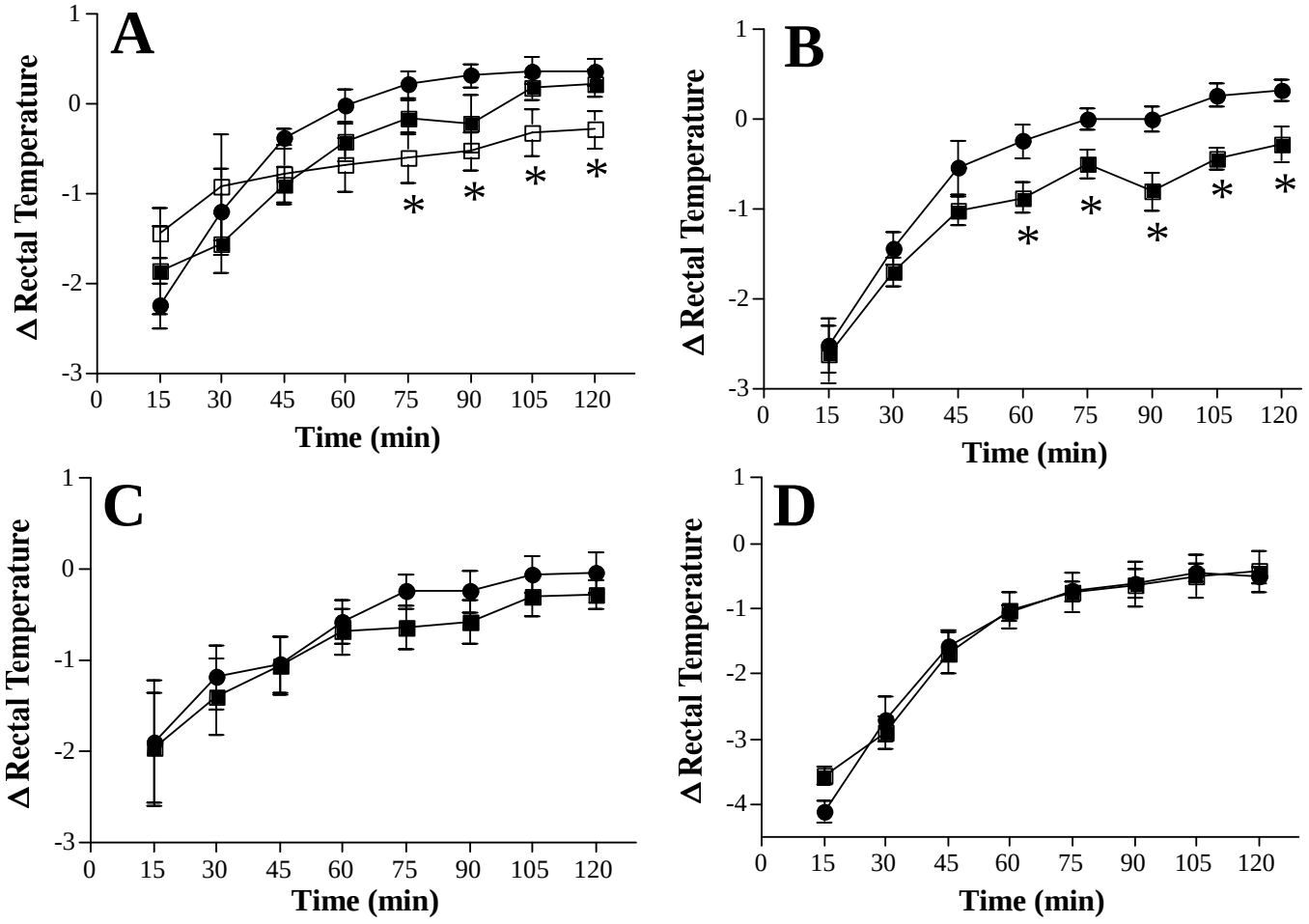


Figure 6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D
Δ Rectal Temperature
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60
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105
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-2
-3
-4
*