

# Relative Timing Between Kappa Opioid Receptor Activation and Cocaine Determines the Impact on Reward and Dopamine Release

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Negative affective states can increase the rewarding value of drugs of abuse and promote drug taking. Chronic cocaine exposure increases levels of the neuropeptide dynorphin, an endogenous ligand at kappa opioid receptors (KOR) that suppresses dopamine release in the nucleus accumbens (NAc) and elicits negative affective states upon drug withdrawal. However, there is evidence that the effects of KOR activation on affective state are biphasic: immediate aversive effects are followed by delayed increases in reward. The impact of KOR-induced affective states on reward-related effects of cocaine over time is not known. We hypothesize that the initial aversive effects of KOR activation increase, whereas the delayed rewarding effects decrease, the net effects of cocaine on reward and dopamine release. We treated rats with cocaine at various times (15 min to 48 h) after administration of the selective KOR agonist salvinorin A (salvA). Using intracranial self-stimulation and fast scan cyclic voltammetry, we found that cocaine-induced increases in brain stimulation reward and evoked dopamine release in the NAc core were potentiated when cocaine was administered within 1 h of salvA, but attenuated when administered 24 h after salvA. Quantitative real-time PCR was used to show that KOR and prodynorphin mRNA levels were decreased in the NAc, whereas tyrosine hydroxylase and dopamine transporter mRNA levels and tissue dopamine content were increased in the ventral tegmental area 24 h post-salvA. These findings raise the possibility that KOR activation—as occurs upon withdrawal from chronic cocaine—modulates vulnerability to cocaine in a time-dependent manner.

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## INTRODUCTION

Baseline emotional states influence the rewarding and reinforcing impact of drugs of abuse (Koob and Le Moal, 2008). The interdependence of affect and addiction is exemplified in the high degree of comorbidity between drug addiction and mood disorders (Grant *et al*, 2004). Drugs of abuse produce profound changes in mood, which has been clearly articulated in the negative reinforcement model of drug addiction: chronic drug-induced negative affective states such as stress, depression, and anxiety enhance the valence (emotional value) of drug reward, thus promoting further drug taking (Koob and Le Moal, 2008). By extension, the opponent process theory of motivation posits that not only do rewarding stimuli trigger counter-adaptations that oppose reward, but aversive stimuli produce rewarding after-effects (Solomon and Corbit, 1974). Here, we explore

the idea that the perceived rewarding impact of drugs of abuse may depend on the timing between a rewarding or aversive stimulus and drug administration.

The neuropeptide dynorphin, an endogenous ligand at kappa opioid receptors (KORs), is expressed within neural circuits that regulate affective state, including the mesocorticolimbic system (Knoll and Carlezon, 2010). KOR activation produces depressive-like and anxiogenic states (Bals-Kubik *et al*, 1993; Pliakas *et al*, 2001; Ebner *et al*, 2010), whereas KOR blockade or genetic deletion of KORs has antidepressant-like and anxiolytic effects (Pliakas *et al*, 2001; Newton *et al*, 2002; Mague *et al*, 2003; Knoll *et al*, 2007; Van't Veer and Carlezon, 2013). Evidence suggests that the neurobiological substrates for the depressive-like effects of KOR activation include suppression of dopamine release in the nucleus accumbens (NAc), a brain region important for reward processing (Spanagel *et al*, 1992; Carlezon *et al*, 2006; Ebner *et al*, 2010). One well-documented neuroadaptation to stress and drugs of abuse is an increase in the activity of KORs within the mesocorticolimbic system (Shippenberg *et al*, 2007; Knoll and Carlezon, 2010).

In drug-dependent humans and animals, dynorphin expression is coincident with the emergence of depressive-like states

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(Hurd and Herkenham, 1993; Shippenberg *et al*, 2007), suggesting that KOR activation contributes to negative reinforcement mechanisms driving continued drug taking and relapse (McLaughlin *et al*, 2006; Bruchas *et al*, 2010; Schindler *et al*, 2010). KOR agonists can increase the behavioral and neurochemical effects of psychostimulants, depending on the timing and context of KOR agonist exposure (Heidbreder *et al*, 1998; Fuentealba *et al*, 2006; Chartoff *et al*, 2008; Ehrich *et al*, 2014). Treatment of rhesus monkeys with the KOR agonist U-50,488 increases cocaine choice (Negus, 2004), suggesting a KOR-mediated increase in the relative reinforcing effects of cocaine. Stress-induced dynorphin release (McLaughlin *et al*, 2003) or pretreatment with KOR agonists (McLaughlin *et al*, 2006) can facilitate cocaine-induced place preferences, and activation of KORs can reinstate cocaine seeking (Redila and Chavkin, 2008). Conversely, KOR antagonists attenuate cocaine withdrawal-induced negative affective states (Chartoff *et al*, 2012), prevent stress-induced reinstatement of drug-seeking behavior (Beardsley *et al*, 2005; Aldrich *et al*, 2009), and attenuate drug self-administration under conditions of dependence (Walker and Koob, 2007; Wee *et al*, 2009).

The purpose of the present study was to test the hypothesis that the aversive effects of acute KOR activation engender neuroadaptations over time that subsequently increase reward function and weaken the rewarding valence of cocaine. We measured the effects of a cocaine challenge on brain stimulation reward using intracranial self-stimulation (ICSS) and—in separate rats—evoked dopamine release in the NAc core using fast scan cyclic voltammetry (FSCV) at various time points (0–48 h) after salvA. Importantly, a recent study used FSCV to demonstrate that an early effect of the KOR agonist U50,488 is a potentiation of stimulated dopamine release in the NAc (Ehrich *et al*, 2014). ICSS is an operant paradigm that measures increases or decreases in reward sensitivity in real time. Treatments that induce rewarding states in humans decrease the frequency of stimulation required to maintain responding (thresholds), whereas treatments with depressive effects increase stimulation thresholds (Carlezon and Chartoff, 2007). Previously, we found that stimulation thresholds are increased immediately after an injection of the potent and highly selective KOR agonist salvinorin A (salvA) (Ebner *et al*, 2010), but decreased 24 h later (Potter *et al*, 2011). The underlying mechanisms for this rebound effect on reward function are not known, but may be triggered by inhibitory actions of KORs on dopamine release in the NAc (Di Chiara and Imperato, 1988; Ebner *et al*, 2010) and subsequent neuroadaptations in dopamine transmission. To explore potential cellular mechanisms underlying KOR-induced opponent processes, we measured KOR-related gene expression and dopamine tissue levels 24 h after a single salvA injection.

## MATERIALS AND METHODS

### Animals

A total of 236 male Sprague-Dawley rats (Charles River Laboratories) weighing approximately 350–400 g at the time of testing were used for these studies. Rats used for ICSS ( $n = 45$ ) and for molecular studies ( $n = 130$ ) were maintained at the McLean Hospital. Rats used for FSCV ( $n = 61$ ) were

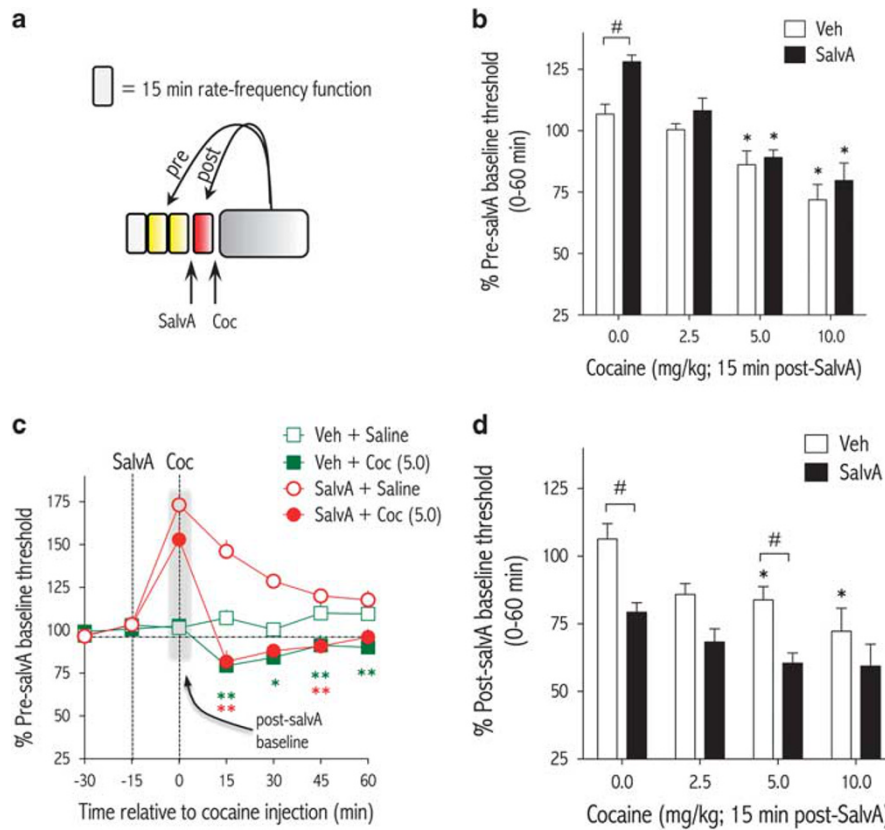
maintained at the University of Illinois at Chicago (UIC). Rats used in ICSS and FSCV studies were individually housed so that head cap assemblies would not be damaged. Rats used in molecular experiments were housed in groups of four. Rats were maintained on *ad libitum* food and water on a 12 h/12 h light/dark cycle, and all experiments were conducted during the light phase. All rats were treated according to the guidelines recommended by the Animal Care Committee at the University of Illinois at Chicago and Institutional Animal Care and Use Committee of McLean Hospital, in strict accordance with the National Institutes of Health's Guide for the Care and Use of Laboratory Animals (1996).

### Drugs

SalvA was provided by Dr Cécile Béguin (McLean Hospital, Belmont, MA). The drug was extracted and purified according to established methods (Béguin *et al*, 2008). SalvA was dissolved in a vehicle of 75% dimethyl sulfoxide (DMSO) plus 25% distilled water (vehicle), and administered intraperitoneally (i.p.) at a dose of 2.0 mg/kg/d in a volume of 1 ml/kg, as in Chartoff *et al* (2008). Cocaine (Mallinckrodt) was dissolved in 0.9% saline and administered i.p. The dose range (0.0–10.0 mg/kg in 1 ml/kg) is based on a previous ICSS study (Tomasiewicz *et al*, 2008).

**Intracranial self-stimulation.** Rats were implanted with stainless steel monopolar electrodes (0.25 mm diameter; Plastics One, Roanoke, VA) aimed at the medial forebrain bundle (MFB) at the level of the lateral hypothalamus (2.8 mm posterior to bregma, +1.7 mm lateral to midline, and 7.8 mm below dura). After 1 week of recovery from surgery, rats were trained to respond for brain stimulation in standard experimental operant procedure chambers (Med Associates, St Albans, VT) using a continuous reinforcement schedule (FR1). Procedures have been described in detail elsewhere (Carlezon and Chartoff, 2007; Chartoff *et al*, 2012).

Rats were trained on a series of 15 descending stimulation frequencies (141–28 Hz, in 0.05 log<sub>10</sub> Hz increments). Each series of frequencies comprised 1-min trials at each frequency. Each 1-min trial included a 5-s period during which non-contingent stimuli were given (1 per second) at the next available stimulation frequency, a 50-s test period during which the number of responses was counted, and a 5-s period during which no stimulation was available. Each stimulation train was followed by a 500 ms time out. All responses were counted, regardless of whether they earned stimulation or occurred during a time out. After responding had been evaluated at each of the 15 frequencies, another series of identical frequencies was started. To characterize the functions relating response strength to reward magnitude (rate-frequency function), a least-squares line of best fit was plotted across the frequencies that sustained responding at 20, 30, 40, 50, and 60% of the maximum rate using customized analysis software. The stimulation frequency at which the line intersected the X-axis (theta 0) was defined as the ICSS threshold (see Carlezon and Chartoff, 2007). Rats were trained for an average of 3–4 weeks until mean ICSS thresholds remained stable ( $\pm 10\%$  for four consecutive days).

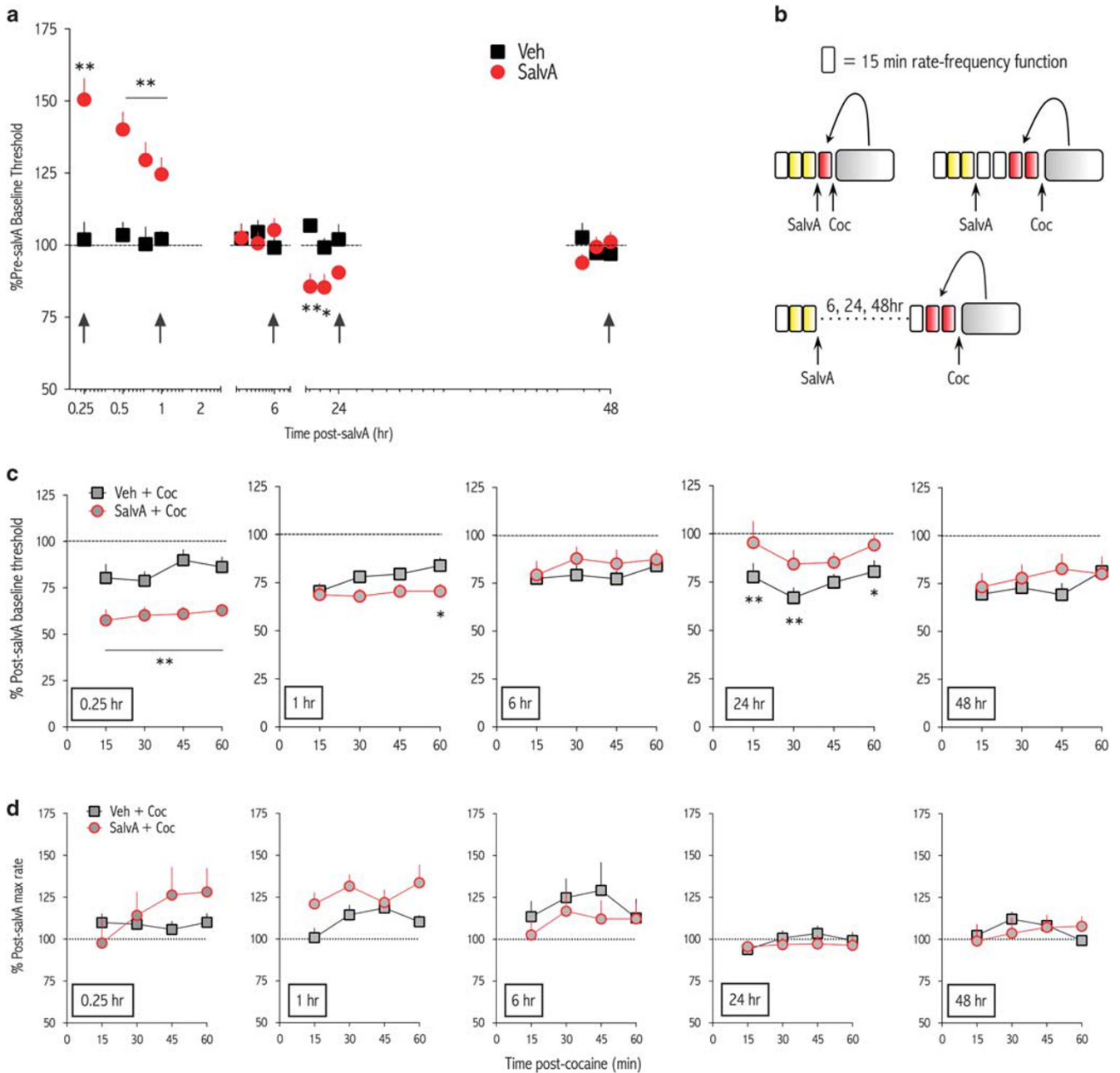


**Figure 1** Baseline affective state impacts the net rewarding effects of cocaine in intracranial self-stimulation (ICSS). (a) To determine the rewarding impact of a cocaine challenge, cocaine-mediated decreases in stimulation thresholds can be compared with pre- or post-salvA baseline thresholds. Yellow boxes represent rate-frequency functions used to calculate pre-salvA baseline thresholds. Red boxes represent rate-frequency functions used to calculate post-salvA baseline thresholds. (b) Cocaine dose-dependently decreases % pre-salvA baseline thresholds in rats treated with salvA (0.0, 2.0 mg/kg) 15 min prior to cocaine (0.0–10.0 mg/kg). (c) Time course of changes in % pre-salvA baseline thresholds in rats treated with salvA (0.0, 2.0 mg/kg) 15 min prior to cocaine (0.0, 5.0 mg/kg). Cocaine has similar effects on ICSS thresholds in rats pretreated with salvA or vehicle. (d) Cocaine-mediated decreases in % post-salvA baseline thresholds are greater in salvA- compared with veh-pretreated rats. \* $P < 0.05$ , \*\* $P < 0.01$  compared with cocaine (0.0 mg/kg) of corresponding veh or salvA pretreatment; # $P < 0.05$  comparing groups under bars ( $n = 12$ ). coc, cocaine; SalvA, salvinorin A; Veh, vehicle (75% DMSO in water).

For drug testing, three rate-frequency functions ( $3 \times 15$  min) were determined for each rat immediately before salvA (or 75% DMSO vehicle) injection (see Figures 1a and 2b). The ICSS thresholds and maximum rates for the second and third functions were averaged and served as 'pre-salvA' baseline parameters. Each rat then received an i.p. injection of salvA (2.0 mg/kg) or vehicle followed by a delay of 15 min, 1, 6, 24, or 48 h prior to a cocaine challenge. In order to obtain a 'post-salvA' (ie, pre-cocaine) baseline for each time point, rate-frequency curves were also generated just prior to cocaine delivery (see Figure 2b). Immediately after cocaine administration, rats were placed back in the ICSS boxes and four additional rate-frequency curves (1 h) were measured. For each salvA pretreatment time, a separate cohort of rats was used and all treatments were administered in random order in a within-subjects design. Drug treatment days were separated by two to three non-drug days in which the rats were exposed to six 15-min rate frequency functions to ensure that they had recovered from prior treatment and their ICSS thresholds remained within 10% of their original baseline values.

**Fast scan cyclic voltammetry.** Carbon fiber recording electrodes were lowered into the NAc core through surgically implanted guide cannulae (1.3 mm anterior to bregma, +1.5 mm lateral to midline, 2.5 mm ventral from skull surface) following established procedures (Ebner *et al*, 2010). A bipolar stimulating electrode (0.20 mm diameter; Plastics One, Roanoke, VA) was positioned dorsal to the ventral tegmental area (VTA) (5.2 mm posterior to bregma, +1.0 mm lateral to midline, 7.0 mm ventral to skull surface) and lowered in 0.2 mm increments until electrically (60 pulses, 60 Hz, 120  $\mu$ A, 4 ms/phase) evoked dopamine release was detected via the carbon fiber electrode. After optimizing evoked dopamine release, the stimulating electrode was cemented and the carbon fiber electrode was removed and replaced with an obdurator (Ebner *et al*, 2010). Rats recovered when they reached pre-surgery body weight (~2–5 days).

**Recordings.** FSCV measures extracellular concentrations of electroactive species including dopamine with subsecond temporal resolution and has been described in detail elsewhere (Fortin *et al*, 2015). Briefly, a fresh carbon fiber microelectrode is lowered into the NAc and held at  $-400$  mV



**Figure 2** Relative timing of salvia pretreatment to cocaine challenge determines whether KOR activation increases or decreases the net rewarding effects of cocaine in ICSS. (a) Time course of salvia (0.0 or 2.0 mg/kg) on ICSS thresholds. Data are reported as % pre-salvia baseline thresholds  $\pm$  SEM and represent four cohorts of rats tested at different time points after salvia (0.0, 2.0 mg/kg) injection: 0–0.25 h, 0.25–1.0 h, 5.25–6.0 h, 23.25–24.0 h, 47.25–48.0 h. (b) Diagram depicting how % baseline stimulation thresholds and maximum rates of responding are calculated. Yellow boxes represent rate-frequency functions used to calculate pre-salvia baseline thresholds. Red boxes represent rate-frequency functions used to calculate post-salvia baseline thresholds. Stimulation thresholds collected after cocaine injection (grey boxes) are divided by post-salvia baseline thresholds and multiplied by 100 to obtain % post-salvia baseline thresholds. (c) Time course of cocaine-induced changes in % post-salvia baseline thresholds, or (d) max rates of responding, in rats treated with salvia (0.0, 2.0 mg/kg) 0.25, 1, 6, 24, or 48 h prior to cocaine (0.0, 5.0 mg/kg). Salvia pretreatment times (0.25, 1, 6, 24, and 48 h) are indicated for each graph ( $n = 12, 10, 6, 11, \text{ and } 6$ , respectively). \* $P < 0.05$ , \*\* $P < 0.01$  comparing veh- and salvA-treated rats.

relative to the Ag/AgCl reaction at the reference electrode. Periodically, the voltage is increased to +1300 mV and decreased to -400 mV in a triangular manner (400 V/s). This triangle waveform is repeated at 10 Hz. Chemical species that are electroactive within the voltage range will oxidize and reduce at various points along the triangle waveform and the resultant currents are measured at the surface of the electrode and recorded on a computer using

software written in LabView (National Instruments, Schaumburg, IL) (Heien *et al*, 2004). The current arising from the double-layer capacitance and electroactivity of surface functional groups on the carbon fiber was removed by background subtraction. The background period (1000 ms) was obtained at 2.5 s before each stimulation of the VTA. Dopamine is electroactive within the applied voltage range, is identified by its oxidation (at  $\sim 0.6$  V) and

**Table 1** qRT-PCR Primers

Gene Abbr.	Gene Name	Genbank #	Forward	Reverse	size (bp)
Dyn	<i>Pdyn</i>	NM_019374	CGCAAATACCCCAAGAGGAG	GCAGGAAGCCCCCATAGC	109
KOR	<i>Oprk1</i>	NM_017167	CTCCCAGTGCCTTGCCTACTC	AGATGTTGGTTGCGGTCTTC	240
Gai2	<i>Gnai2</i>	NM_031035	GATGATCGACAAGAACCTGCGGG	CAGGTTGCCCATGGCTTTGACG	223
Grk3	<i>Adrbk2</i>	NM_012897	TACGGAAGTACCCGCCACCCC	GTTTCGGCATTGACGGCGTCG	211
TH	<i>Th</i>	NM_012740	TGAAGGCTTATGGTGCAGGGC	AATGGGCGCTGGATACGAGAGG	211
DAT	<i>Slc6a3</i>	NM_012694	GGAAGCTGGTCAGCCCCTGCTT	GAATTGGCGCACCTCCCCTCTG	266
$\beta$ -actin	<i>Nba</i>	NM_031144	AGGGAAATCGTGCCTGACAT	AAGGAAGGCTGGAAGAGAGC	182
GTFIIb	<i>Gtf2B</i>	NM_031041	TGCGATAGCTTCTGCTTG TC	TCAGATCCACGCTCGTCTC	155
GFAP	<i>Gfap</i>	NM_017009	GCAGTGGCCACCAGTAACATGC	TCCTCCTGTTGCGGCATTTGCC	215

reduction (at  $\sim -0.2$  V vs Ag/AgCl) features, and is quantified by measuring its oxidative current, which is directly proportional to its concentration at the electrode surface (Fortin *et al*, 2015). Current is converted into concentration by means of calibrating the carbon fiber electrode after its use in a flow injection system with a known concentration of dopamine.

### Histology

After completion of the FSCV experiments, rats were injected with a lethal dose of sodium pentobarbital (100 mg/kg). To verify the placement of the recording electrode in the NAc, an epoxy-insulated stainless steel microelectrode (A-M Systems) was lowered into the NAc to the depth where experimental recordings were made and an electrolytic lesion (0.5 mA, 4 s) was made. Following the lesion, rats were transcardially perfused with 0.9% saline followed by a 4% formalin solution. Coronal sections (50  $\mu$ m) through the NAc were made. The tissue was stained with cresyl violet and examined under a light microscope (VistaVision) for the location of the lesion. Placement of the recording electrode within the NAc was confirmed using Paxinos and Watson (Paxinos and Watson, 1996).

### High Performance Liquid Chromatography

Rats were killed 15 min or 24 h after salvA (0.0, 2.0 mg/kg) injection by rapid decapitation, and brains were rapidly removed, frozen, and stored at  $-80$  °C. Frozen brains were coronally sectioned on a cryostat (HM505E, Microm, Walldorf, Germany,  $-20$  °C) until reaching the NAc. Bilateral 1-mm<sup>3</sup> punches of the NAc core were made. Brains were sectioned further until reaching the VTA and bilateral 1-mm<sup>3</sup> punches were taken. Tissue punches were sonicated in 100  $\mu$ l of tissue buffer (0.05 M sodium phosphate/0.03 M citric acid, 25% methanol (v/v), pH 2.5), and centrifuged twice at 22 000 g, as in Son *et al* (2011). Protein content of each sample was determined (Pierce BCA Protein Reagent Kit; Rockford, IL). Samples (10  $\mu$ l) were assayed for dopamine and its metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) using high-pressure liquid chromatography with electrochemical detection. Samples were loaded on a microbore analytical column 5  $\mu$ m, C18, 150  $\times$  1 mm UniJet SepStik (BASi, West Lafayette, IN) for separation. Quantities

of dopamine and DOPAC were measured on a 3-mm glassy carbon-wired electrode operated at +750 mV vs an Ag/AgCl reference electrode. The mobile phase consisted of 25 mM NaH<sub>2</sub>PO<sub>4</sub>, 50 mM sodium citrate, 27  $\mu$ M EDTA, 10 mM diethylamine HCl, and 2.2 mM 1-octanesulfonic acid sodium salt at a pH of 3.2 with H<sub>3</sub>PO<sub>4</sub>. Methanol (30 ml) and dimethylacetamide (22 ml) were added per 1 l of Millipore water. After mixing, the solution was degassed for 20 min. The mobile phase was delivered at a rate of 70  $\mu$ l/min by a solvent delivery system (Shimadzu, Kyoto, Japan). Dopamine and DOPAC peaks were quantified by comparison with peak heights of four standard solutions each with a minimum R<sup>2</sup> of 0.99. The detection limit was 1 fmol.

### Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Naïve male rats were treated with salvA (2.0 mg/kg) or vehicle (75% DMSO) and returned to their home cages. Twenty-four hours later, rats were killed by decapitation and brains removed and immediately frozen in isopentane kept over dry ice. Bilateral tissue punches of the NAc and VTA were taken as described above for high performance liquid chromatography. RNA extraction, cDNA synthesis, and qRT-PCR were performed as described (Pravetoni *et al*, 2014). Briefly, RNA was extracted using PureLink RNA Mini Kit (Invitrogen). RNA quality and quantity were assessed using an RNA 6000 Nano Chip (Agilent, Santa Clara, CA) on an Agilent Bioanalyzer 2100. One microgram of total RNA was used to synthesize cDNA using iScript cDNA Synthesis Kit (Bio-Rad) in a ThermoHybaid iCycler (Thermo Scientific). Primers were designed using NCBI Primer-BLAST (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/>) and purchased from Integrated DNA Technologies (Coralville, IA). See Table 1 for a list of primer sequences. Melt curve analysis and polyacrylamide gel electrophoresis confirmed the specificity of the primers.

A Q-PCR kit (iQ SybrGreen Supermix, Bio-Rad) was used. The reaction was carried out on a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad) in a volume of 20  $\mu$ l, with 2  $\mu$ l of 3 mM forward and reverse primers and 4  $\mu$ l cDNA sample diluted 1 : 10. PCR cycling conditions were 95 °C for 5 min; 40 cycles at 94 °C for 15 s, 60 °C for 15 s, 72 °C for 15 s. Data were collected at a read temperature of 84 °C for 15 s based on the amplicon melt temperatures. For

each brain region, standard dilution curves were generated for each primer set by serially diluting (1.00-, 0.25-, 0.0625-, and 0.0156-fold) a master cDNA stock comprising an equal mix of cDNA from the two treatment groups. The  $\log_{10}$  of the dilution values was plotted against the threshold cycle values for the standard curves. MyiQ Optical System Software (Bio-Rad) was used to analyze the data. Samples containing no cDNA template and samples from cDNA reactions containing no reverse transcriptase were run as controls for contamination and amplification of genomic DNA, respectively. Reported values were normalized to the average values of the internal standards  $\beta$ -actin, Gtf2b, and Gfap for each sample. Data are expressed as mean relative levels of [gene of interest/internal standard average]  $\pm$  SEM.

### Western Blot Analysis

Naïve male rats were treated with salvA (2.0 mg/kg) or vehicle (75% DMSO), returned to their home cages, and killed 24 h later by decapitation. Tissue punches of the NAC and VTA were taken as described above for qRT-PCR. SDS polyacrylamide gel electrophoresis and western blot analysis were done as described (Cone *et al*, 2013). Nonspecific binding sites on the polyvinylidene fluoride membrane protein immunoblots were blocked for 2 h at room temperature in blocking buffer (5% nonfat dry milk in PBS and 0.1% Tween 20 (PBS-T)). Blots were then incubated in primary antibody in PBS-T overnight at 4°C. Primary antibodies used were: 1 : 10 000 anti-TH (Millipore), 1 : 2000 anti-phospho Serine-31 tyrosine hydroxylase (TH) (Abcam), 1 : 5000 rabbit polyclonal anti-DAT (Millipore), and 1 : 20 000 rabbit polyclonal anti-actin (Sigma). Blots were washed 4  $\times$  5 min in PBS-T and then incubated in secondary antibody (1 : 5000 goat anti-rabbit (or anti-mouse) horseradish peroxidase-linked IgG (Vector Laboratories, Burlingame, CA)) for 2 h at room temperature. Blots were washed 4  $\times$  5 min in PBS-T, followed by immunological detection using Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). SeeBlue Plus 2 (Invitrogen) pre-stained standards were run for molecular weight estimation.

Protein immunoblots were analyzed using Kodak 1D Image Analysis software (Kodak, Rochester, NY). Relative optical densities were determined for each band of interest (TH and pTH at 60 kDa, DAT at 74 and 54 kDa, and  $\beta$ -actin at 43 kDa). To control for loading differences of protein, the optical density of each band was normalized with the corresponding optical density of  $\beta$ -actin. To allow for comparisons between blots, data were normalized to the vehicle-treated controls in each experiment. Data are expressed as the mean fold induction compared with vehicle control  $\pm$  SEM.

### Statistical Analysis

The time course of drug effects on ICSS parameters (thresholds, maximum rates of responding) was analyzed using two-way (treatment  $\times$  time) ANOVAs with repeated measures on both factors. The time course of drug effects on dopamine release was analyzed using two-way (treatment  $\times$  time) ANOVAs. Effects of salvA on cocaine dose response, gene and protein expression were analyzed using two-way (treatment  $\times$  dose/gene) ANOVAs with repeated measures

on cocaine dose, gene, and protein. The effect of salvA on tissue dopamine content was analyzed using two-way (treatment  $\times$  time) ANOVAs. All significant effects and interactions were analyzed further using Bonferroni/Dunn's correction tests.

## RESULTS

### Baseline Stimulation Threshold Determines the Magnitude of Cocaine-Mediated Increases in the Reinforcing Efficacy of ICSS

Rats were treated with salvA (0.0, 2.0 mg/kg) and ICSS behavior measured for 15 min (1 rate-frequency function). Rats were then treated with cocaine (0.0, 2.5, 5.0, 10.0 mg/kg) and ICSS behavior measured for 1 h. Post-cocaine changes in ICSS thresholds were compared with pre-salvA baseline thresholds (Figure 1a and b). The effect of a 15-min salvA pretreatment on cocaine-induced decreases in ICSS thresholds depended on salvA ( $F_{1,11} = 5.08$ ,  $p < 0.05$ ) and cocaine dose ( $F_{3,33} = 38.47$ ,  $p < 0.0001$ ) (Figure 1b). Bonferroni's *post hoc* analyses revealed that ICSS thresholds only differed between salvA and vehicle pretreatments at the 0.0 mg/kg cocaine dose. This is expected, as salvA on its own increases ICSS thresholds (Ebner *et al*, 2010). In both veh- and salvA-pretreated rats, cocaine at 5.0 and 10.0 mg/kg significantly decreased ICSS thresholds compared to veh+coc (0.0 mg/kg).

To more clearly visualize the effect of salvA pretreatment on cocaine-mediated decreases in ICSS thresholds, Figure 1c shows the time course of changes in percent pre-salvA baseline thresholds after salvA (2.0 mg/kg) and cocaine (5.0 mg/kg) treatments. The effect of salvA and cocaine on ICSS thresholds depends on a main effect of treatment ( $F_{3,33} = 27.63$ ,  $p < 0.0001$ ), a main effect of time ( $F_{6,66} = 30.74$ ,  $p < 0.0001$ ), and an interaction between treatment and time ( $F_{18,198} = 15.84$ ,  $p < 0.0001$ ). *Post hoc* tests demonstrate that cocaine significantly decreases ICSS thresholds compared with saline-treated rats, regardless of salvA pretreatment.

Given that salvA changes baseline affective states, we explored how post-salvA anhedonic baselines altered the rewarding effects of cocaine. We compared post-cocaine stimulation thresholds with post-salvA thresholds (Figure 1a), generating a 'delta' in thresholds that takes into account salvA-induced anhedonia. Using this method of analysis, the effects of salvA on cocaine reward depend on salvA pretreatment ( $F_{1,11} = 42.67$ ,  $p < 0.001$ ) and cocaine dose ( $F_{3,33} = 5.98$ ,  $p < 0.01$ ; Figure 1d). *Post hoc* tests show that cocaine dose-dependently decreases ICSS thresholds in vehicle-, but not salvA-, pretreated rats. Furthermore, the cocaine-induced decrease in thresholds from post-salvA baseline is larger in rats pretreated with salvA compared with vehicle-pretreated rats.

### Temporal, Biphasic Effects of salvA on ICSS

Previously, we demonstrated that salvA has biphasic effects on brain stimulation reward. Initially, salvA produces a robust increase in ICSS thresholds that are followed, 24-h later, with a decrease in thresholds (Potter *et al*, 2011). We replicate and extend that finding here by examining the temporal effects of salvA on ICSS thresholds over the course of 48 h post-salvA injection. Separate groups of rats were

used for each time segment post-salvA (15 min, 1, 6, 24, 48 h). Figure 2a shows a composite of post-salvA (or vehicle) thresholds relative to pre-salvA (or vehicle) thresholds for each time segment. Separate statistical analyses were carried out for each time segment. There were significant effects of treatment at the 15 min, 1, and 24 h time points (15 min:  $t$ -test,  $t=5.30$ ,  $p<0.01$ ; 1 h:  $F_{1,8}=36.72$ ,  $p<0.01$ ; 24 h:  $F_{1,10}=22.69$ ,  $p<0.01$ ). *Post hoc* tests showed that, compared with vehicle (75% DMSO), salvA resulted in significantly increased ICSS thresholds for the first 60 min and significantly decreased ICSS thresholds 24 h after treatment. Thresholds were normalized by 48 h.

### Effect of salvA on Cocaine-Mediated Decreases in ICSS Thresholds Depends on Time Interval between salvA and Cocaine Administration

Next, we measured the magnitude of cocaine-induced decreases in ICSS thresholds 15 min, 1, 6, 24, and 48 h after a single salvA (2.0 mg/kg) injection. For each time point, post-cocaine ICSS thresholds were compared with post-salvA (eg, pre-cocaine) baseline thresholds (see Figure 2b). Cocaine-induced changes in ICSS thresholds at 15 min, 1, and 24 h depended on salvA pretreatment (Figure 2c). When salvA was administered 15 min or 1 h prior to cocaine, the percent changes in thresholds from post-salvA baseline thresholds were significantly greater in rats pretreated with salvA compared with vehicle (Figure 2c: 0.25 h:  $F_{1,11}=18.02$ ,  $p<0.001$ ; Figure 2c: 1 h:  $F_{1,9}=5.64$ ,  $p<0.05$ ). In contrast, when salvA was administered 24 h prior to cocaine, the percent change in thresholds from post-salvA baseline thresholds was significantly less in rats pretreated with salvA compared with vehicle (Figure 2c: 24 h  $F_{1,10}=5.68$ ,  $p<0.05$ ). Cocaine-induced decreases in ICSS thresholds were similar in rats treated 48 h earlier with salvA or vehicle. In contrast to effects on ICSS thresholds, which are a measure of reward sensitivity, salvA pretreatment did not affect maximum rates of responding in response to cocaine at any time point (Figure 2d). Time was a significant factor in the effects of cocaine on rates of responding in the 15-min post-salvA group ( $F_{3,33}=8.53$ ,  $p<0.001$ ; Figure 2d, 0.25 h).

### Effect of salvA on Cocaine-Mediated Increases in Evoked Dopamine Release Depends on Time Interval Between salvA and Cocaine Administration

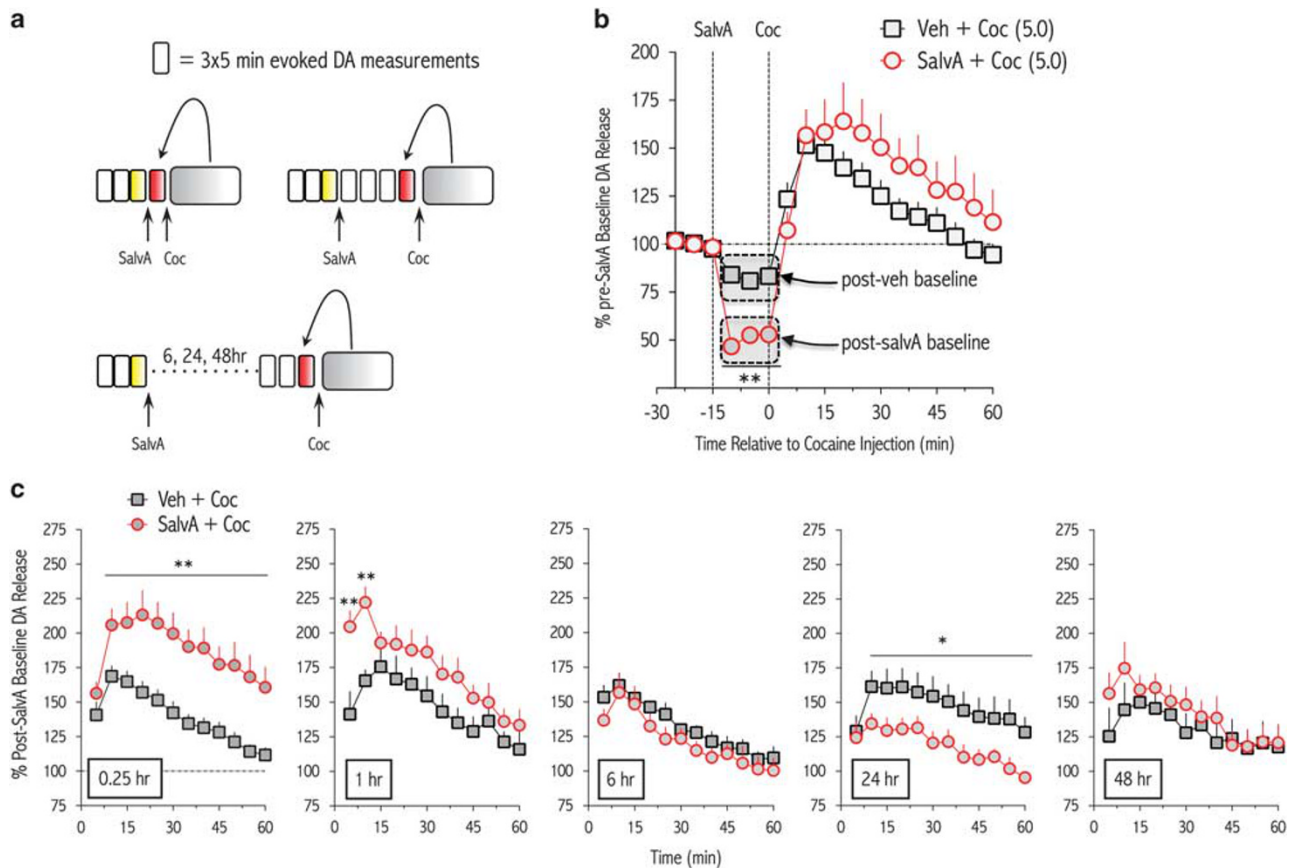
This study examined the temporal effects of acute salvA (2.0 mg/kg) on the magnitude of cocaine-potentiated dopamine release in the NAc core evoked by VTA stimulation. All data were normalized for each subject to the average of the baseline measurements (percent baseline) to control for differences in the initial magnitude of evoked dopamine release across rats. We have previously reported that salvA causes a rapid and robust suppression of stimulated dopamine release in the NAc core (Ebner *et al*, 2010). To start, we determined the effect of a 15-min salvA pretreatment on cocaine-potentiated dopamine release: rats were treated with salvA (2.0 mg/kg) 15-min prior to cocaine (5.0 mg/kg) (Figure 3b). Stimulated dopamine release was measured prior to any injection, and data collected in the 1 5-min prior to salvA were used as pre-salvA baseline (see schematic, Figure 3a). Stimulated dopamine release

was measured for 15 min immediately after salvA injection, and these data were used as post-salvA baseline, and for 1 h immediately after cocaine injection. When the magnitude of evoked dopamine release is compared with pre-salvA baseline, there is a significant main effect of time ( $F_{17,170}=33.85$ ,  $p<0.0001$ ), but not treatment ( $F_{1,10}=0.28$ , *ns*), and a significant interaction between treatment and time ( $F_{17,170}=4.54$ ,  $p<0.0001$ ; Figure 3b). *Post hoc* analysis revealed that salvA significantly decreased dopamine levels compared with vehicle in the first 15 min post-salvA injection.

We next measured the magnitude of cocaine-induced increases in stimulated dopamine release 15 min, 1, 6, 24, and 48 h after a single salvA (2.0 mg/kg) injection (Figure 3c). For each time point, the magnitude of post-cocaine dopamine was compared with post-salvA (eg, pre-cocaine) baseline levels. For the 15-min salvA pretreatment, the percent change from post-salvA baseline in cocaine-stimulated dopamine release depended on an interaction between treatment and time ( $F_{11,55}=2.44$ ,  $p<0.05$ ). *Post hoc* analysis showed that the potentiation of evoked dopamine release by cocaine from rats treated with salvA and cocaine was significantly greater than that from vehicle pre-treated rats at every time point except the first 5 min post-cocaine. For the 1- and 24-h salvA pretreatment groups, the percent change from post-salvA baseline in cocaine-stimulated dopamine release depended on time (1 h:  $F_{11,55}=12.39$ ,  $p<0.0001$ ; 24 h:  $F_{11,66}=11.50$ ,  $p<0.0001$ ). *Post hoc* analysis of the 1-h salvA pretreatment group showed that cocaine-potentiated dopamine release from rats treated with salvA and cocaine was significantly greater than dopamine release from vehicle pre-treated rats for the first 10 min post-cocaine. In contrast, *post hoc* analysis of the 24-h salvA pretreatment group showed that cocaine-potentiated dopamine release from rats treated with salvA and cocaine was significantly blunted compared with dopamine release from vehicle pre-treated rats during the entire test session. Histological verification of electrode recording sites in the NAc core for each salvA pretreatment time are shown in Figure 4.

### Temporal Effects of salvA on Measures of Dopamine Synthesis

Because salvA had temporally dependent and opposing effects on cocaine-potentiated reward and dopamine release, we used high performance liquid chromatography to measure the tissue content of dopamine and the dopamine metabolite DOPAC in the NAc and VTA 15 min or 24 h after a single salvA (2.0 mg/kg) or vehicle (75% DMSO) injection in a separate group of rats. There was no significant effect of treatment or time on dopamine or DOPAC levels in the NAc (Figure 5a and b). In the VTA, however, there was a significant effect of time on dopamine (Figure 5c;  $F_{1,44}=5.94$ ,  $p<0.05$ ). At the 24-h time point, *post hoc* analysis showed a trend ( $p=0.053$ ) for dopamine content to be higher in the VTA of salvA-treated compared with vehicle-treated rats. Likewise, DOPAC levels in the VTA depended on treatment (Figure 5d;  $F_{1,44}=4.10$ ,  $p<0.05$ ), with *post hoc* tests showing that dopamine content was significantly higher in the VTA of salvA-treated compared with vehicle-treated rats 24 h after salvA treatment.



**Figure 3** Relative timing of salvia pretreatment to cocaine challenge determines whether KOR activation increases or decreases cocaine-potentiated dopamine release in the nucleus accumbens (NAc) core measured with fast scan cyclic voltammetry (FSCV). (a) Diagram depicting how % baseline dopamine release is calculated. Yellow boxes represent 3 × 5-min bins of VTA-stimulated dopamine release measurements taken just prior to salvia treatment (pre-salvia baseline). Red boxes represent 3 × 5-min bins of VTA-stimulated dopamine release measurements taken just prior to cocaine treatment (post-salvia baseline). (b) Time course of the effects of a cocaine (5.0 mg/kg) challenge administered 0.25 h after salvia (0.0, 2.0 mg/kg) on % pre-salvia baseline dopamine release. There is no significant difference in evoked dopamine release after a cocaine challenge between veh- and salvia-pretreated rats. (c) Time course of the effects of a cocaine (5.0 mg/kg) challenge administered 0.25, 1, 6, 24, or 48 h after salvia (0.0, 2.0 mg/kg) on % post-salvia baseline dopamine release. Salvia pretreatment times (0.25, 1, 6, 24, and 48 h) are indicated for each graph ( $n = 6-7$ ). \* $P < 0.05$ , \*\* $P < 0.01$  comparing veh- and salvia-treated rats. VTA, ventral tegmental area.

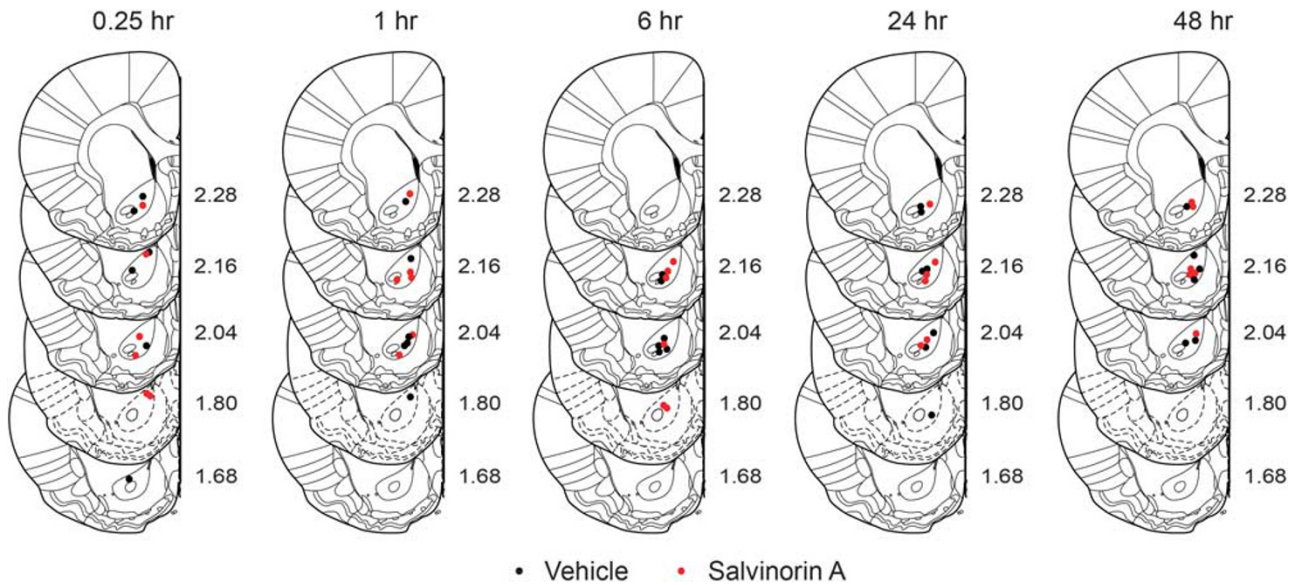
### Effect of salvia on Expression of Dopamine-Related Genes in the NAc and VTA 24 h Post Injection

On the basis of the temporal effects of salvia on behavior and dopamine neurochemistry described above, we hypothesized that delayed (ie, 24 h) effects of salvia would be associated with neuroplastic changes in dopamine-related gene expression in mesolimbic brain regions. We used qRT-PCR to determine whether a single salvia injection changes the expression levels of mRNAs encoding TH and the dopamine transporter (DAT). Tissue from the NAc and VTA was collected from rats 24 h after salvia (2.0 mg/kg) or vehicle (75% DMSO) injection. There was a significant effect of treatment on the dopamine-related genes TH and DAT (Figure 6a;  $F_{(1, 26)} = 12.26$ ,  $p < 0.01$ ), with *post hoc* tests revealing that salvia significantly increased TH and DAT.

To determine whether salvia-mediated increases in TH and DAT mRNA resulted in increased protein expression, separate rats were treated with salvia (2.0 mg/kg) or vehicle (75% DMSO), and tissue from the NAc and VTA was

extracted 24 h later. Protein immunoblots were probed with antibodies specific for DAT, TH, and ERK-dependent phospho-Ser31 TH (pTH<sup>Ser31</sup>). The DAT antibody recognizes both the non-reduced 74 kDa and the reduced 54 kDa forms of the transporter. KORs activate ERK (Bruchas and Chavkin, 2010; Potter *et al*, 2011), and pTH<sup>Ser31</sup> increases dopamine synthesis in the brain (Lindgren *et al*, 2002). In the VTA, protein expression depended on treatment (Figure 6b;  $F_{1,50} = 6.13$ ,  $p < 0.05$ ), with *post hoc* analysis showing a significant increase in pTH expression and a strong trend ( $p = 0.058$ ) towards an increase in TH expression 24-h after salvia compared with vehicle treatment. In the NAc, protein expression depended on an interaction between treatment and protein (Figure 6c;  $F_{2,51} = 3.87$ ,  $p < 0.05$ ). *Post hoc* analysis showed that 24 h after salvia treatment, pTH levels were decreased, with no change in total TH levels. DAT protein exists in multiple states of glycosylation, which results in at least two DAT-specific bands (74 and 54 kDa) on our western blots. The largest molecular weight form of DAT is the most glycosylated and is considered to be the most mature form. Differences in expression levels of any





**Figure 4** Histological verification of recording sites. Electrolytic lesions confirmed that recording sites (●, Vehicle- or ●, Salva-treated rats) were within the nucleus accumbens core. The numbers to the right of individual sections indicate distance, in millimeters, anterior to bregma (Paxinos and Watson, 1996).

of the DAT isoforms could have functional effects on reuptake. However, we did not observe any differences in expression level at any DAT band. Data represented in Figure 6b and c are from the 74 kDa mature DAT band (see Figure 6d and e).

#### Effect of salva on Expression of KOR-Related Genes in the NAcS and VTA 24 h Post Injection

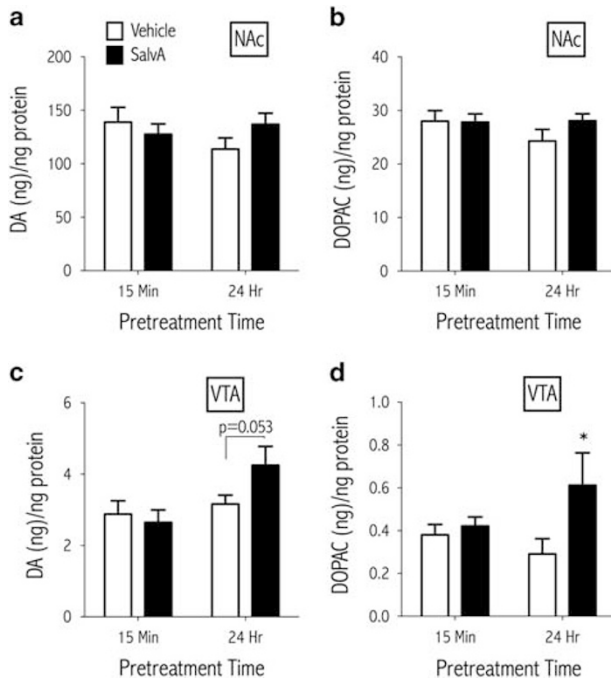
We used qRT-PCR to measure the expression levels of a select number of genes whose protein products have a key role in transducing KOR signaling (dynorphin (Dyn), KOR, G-protein alpha inhibitory subunit 2 (*Gai2*), and G-protein receptor kinase 3 (*Grk3*)) and dopamine signaling (dopamine D2 receptor (D2R)). Messenger RNA levels in the NAc depended on treatment (Figure 7a;  $F_{1,101} = 25.29$ ,  $p < 0.0001$ ), with *post hoc* tests revealing that salva significantly reduced Dyn, KOR, and *Gai2*. In the VTA, there was no effect of treatment on mRNA levels for KOR-related genes or D2Rs (Figure 7b;  $F_{3, 95} = 1.078$ , *ns*).

Given the salva-mediated reductions in KOR signaling genes in the NAc, we hypothesized that the behavioral response to a challenge injection of salva 24 h after the initial injection would be blunted (ie, tolerance). ICSS-trained rats were treated with salva (2.0 mg/kg) and ICSS thresholds measured for 1 h. Twenty-four hours later, rats were again treated with salva (2.0 mg/kg) and ICSS thresholds measured for 1 h. Changes in ICSS thresholds from pre-salva (treatment 1) baseline were dependent on an interaction between treatment and time (Figure 7c;  $F_{9,144} = 2.42$ ,  $p < 0.05$ ). *Post hoc* tests showed that salva significantly increased ICSS thresholds on both days 1 and 2. Although the effect of salva on ICSS thresholds on day 2 was consistently less than the effect on day 1, the difference was not significant, suggesting a lack of overt tolerance to the threshold-increasing effects of salva at a single 24-h inter-injection interval.

#### DISCUSSION

The present study addressed the question of whether KOR activation has biphasic effects on the magnitude of cocaine reward in rats. Consistent with our hypothesis and previous findings (McLaughlin *et al*, 2006; Schindler *et al*, 2010; Ehrich *et al*, 2014), we found that the immediate (0.25–1 h) dysphoric effect of the KOR agonist salva coincides with a net increase in sensitivity to cocaine reward. In contrast, a delayed (24 h) effect of salva is a rebound increase in basal hedonic state that coincides with a net decrease in sensitivity to cocaine reward. These biphasic effects of KOR activation on sensitivity to cocaine reward temporally map onto the effects of KOR activation on cocaine-modulated dopamine release in the NAc core. An immediate effect of salva is a decrease in dopamine release evoked by VTA stimulation that coincides with a net increase in cocaine-potentiated dopamine release, consistent with Ehrich *et al* (2014). In contrast, a delayed effect of salva is an increase in measures of dopamine signaling that coincides with a net decrease in cocaine-potentiated dopamine release. These delayed behavioral and electrochemical effects of KOR activation that are evident 24 h post-salva are correlated with decreased expression of genes encoding KOR, prodynorphin, and the inhibitory *Gai2* G-protein subunit in the NAc and increased expression of genes encoding TH and DAT in the VTA. Taken together with our observation of increased TH protein expression and dopamine tissue content in the VTA, these data raise the possibility that the initial KOR-mediated decreases in NAc dopamine release trigger compensatory adaptations in both KOR- and dopamine-related signaling pathways that result in a delayed emergence of enhanced baseline dopaminergic activity and reward sensitivity.

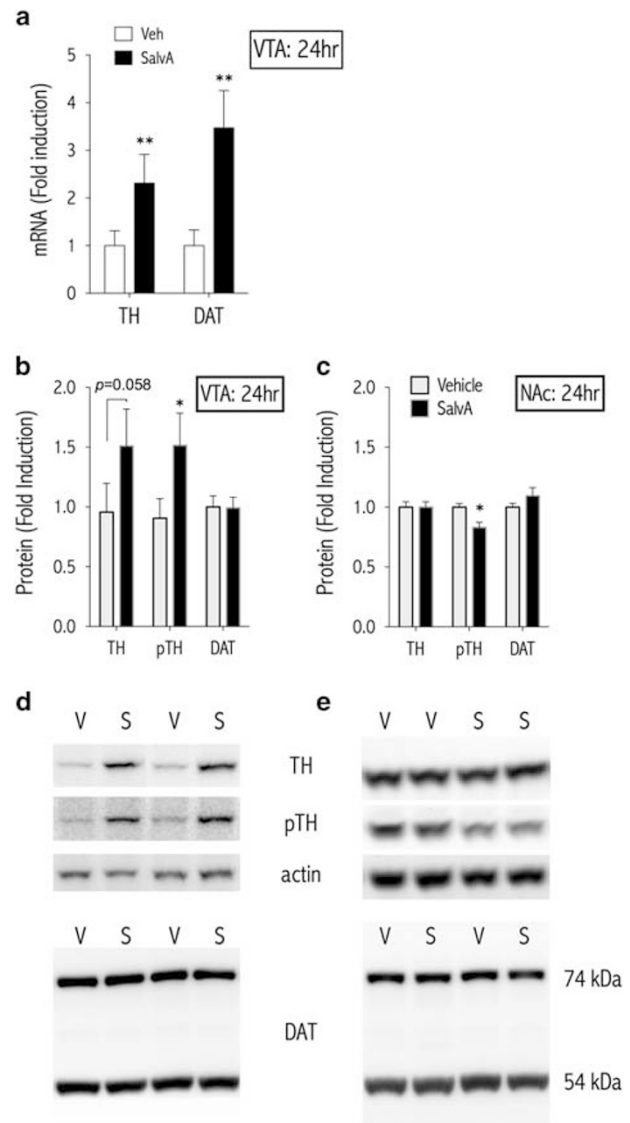
The rewarding properties of cocaine are not fixed, but are modulated by external and internal stimuli including baseline affective state (Koob and Le Moal, 2008; Schindler *et al*, 2010). Although KOR activation can oppose the acute



**Figure 5** Tissue content of dopamine and its metabolite DOPAC in the nucleus accumbens (NAc) and ventral tegmental area (VTA) using high performance liquid chromatography (HPLC). There was no difference in tissue dopamine (a) or DOPAC (b) levels in the NAc 15 min or 24 h after salvA (2.0 mg/kg) treatment. Twenty-four hours after salvA treatment, there was a trend towards an increase in dopamine (c), and a significant increase in DOPAC (d) levels in the VTA ( $n=12$ ), \* $P<0.05$  comparing veh- and salvA-treated rats.

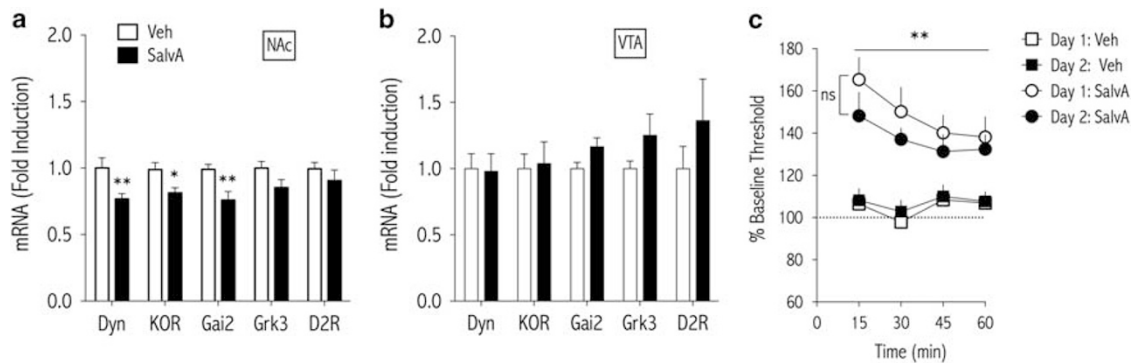
intoxicating effects of cocaine (Negus *et al*, 1997; Schenk *et al*, 1999; Tomasiewicz *et al*, 2008), accumulating evidence suggests that the behavioral consequence of KOR activation most relevant to addiction is negative reinforcement. In this process, response-contingent cessation of an aversive state increases the probability of a subsequent response. In other words, KOR activation promotes drug taking as a means to ameliorate stress- or drug-withdrawal-induced negative affective states. We—and others—argue that the rewarding valence of cocaine is defined by the magnitude of change ( $\Delta$ ) from pre-cocaine to post-cocaine hedonic state (Bruchas *et al*, 2010; Schindler *et al*, 2010; Ehrich *et al*, 2014). Although ICSS does not directly model addiction, our findings are consistent with those showing that KOR activation can increase motivation to seek cocaine and precipitate relapse (Negus, 2004; Beardsley *et al*, 2005; Redila and Chavkin, 2008; Graziane *et al*, 2013). KOR-triggered rebound positive affective states are short-lived, as ICSS thresholds return to pre-salvA baseline levels by 48 h. This is consistent with brain homeostatic regulatory mechanisms, which are reactionary responses to acute stimuli. Conceptually, this idea is the flip of that proposed for hedonic processes associated with intake of drugs of abuse (Koob and Le Moal, 2008).

Using FSCV, we conducted parallel studies of the temporal effects of salvA on cocaine-induced potentiation of evoked dopamine release in the NAc core. We focused on the core because salvA-mediated inhibition of dopamine release in the NAc core is greater than in the shell (Ebner *et al*, 2010).



**Figure 6** Gene and protein expression analysis using western immunoblotting in the nucleus accumbens (NAc) and ventral tegmental area (VTA) of rats 24 h after salvA (0.0, 2.0 mg/kg). (a) TH and DAT mRNA levels are increased in the VTA 24 h after salvA (0.0, 2.0 mg/kg). (b) There is a trend for levels of total TH ( $p=0.058$ ) to be increased, and pTH<sup>Ser31</sup> levels are significantly increased, in VTA tissue punches from salvA-treated compared with veh-treated rats ( $n=8-12$ ). (c) Levels of TH phosphorylated at the extracellular signal regulated kinase-sensitive serine residue 31 (pTH<sup>Ser31</sup>) are reduced in NAc tissue punches from salvA-treated compared with veh-treated rats ( $n=8-12$ ). (d, e) Representative western immunoblot images. \* $P<0.05$ , \*\* $P<0.01$  comparing veh- and salvA-treated rats. DAT, dopamine transporter; S, salvA; TH, tyrosine hydroxylase; V, vehicle.

Importantly, our goal in this study was to isolate the effects of KOR activation on dopamine release dynamics. Therefore, we chose to evoke dopamine release by directly stimulating the VTA, rather than the MFB, which we used for our ICSS studies. It has previously been demonstrated that stimulation of the VTA and MFB with similar trains evokes similar electrochemical responses in the NAc as measured by FSCV (Cheer *et al*, 2004; Cheer *et al*, 2006), and KOR agonists produce similar reductions in dopamine release in the NAc



**Figure 7** Gene expression analysis using qRT-PCR in the nucleus accumbens (NAc) and ventral tegmental area (VTA) of rats 24 h after salvA (0.0, 2.0 mg/kg). Levels of Dyn, KOR, and  $G\alpha_2$ , Grk3, and D2R mRNA are measured in (a) NAc and (b) VTA tissue punches from salvA-treated compared with veh-treated rats (NAc:  $n = 10$ – $12$ ; VTA:  $n = 6$ – $9$ ). (c) Rats are treated with salvA (0.0 or 2.0 mg/kg) on day 1 and again 24 h later on day 2 to test for tolerance. ICSS thresholds are measured immediately after each salvA injection. SalvA significantly increases ICSS thresholds on both days 1 and 2, with no significant difference in responses between the days ( $n = 13$ ). \* $P < 0.05$ , \*\* $P < 0.01$  comparing veh- and salvA-treated rats. Dyn, dynorphin; Grk3, G-protein receptor kinase 3; KOR, kappa opioid receptor.

when either the MFB or the VTA is used to stimulate dopamine neurons (Ehrich *et al*, 2014). However, the effects on dopamine neurons are indirect with MFB stimulation, which comprises over 50 fiber systems (Carlezon and Chartoff, 2007). Our finding that the net increase in dopamine release produced by a cocaine challenge is potentiated immediately after salvA compliments a recent study in mice showing that 1 h after administration of the KOR agonist U50,488, cocaine-potentiated NAc dopamine release evoked by either pedunculo-pontine tegmental nucleus or VTA stimulation is increased (Ehrich *et al*, 2014). These findings are also consistent with previous work showing that KOR agonists can increase stimulated dopamine release in the striatum (Heidbreder *et al*, 1998; Fuentealba *et al*, 2006). Conditions that reduce stimulated dopamine release create a system with low dopamine ‘noise’ (Weiss *et al*, 1992; Volkow *et al*, 1997). Subsequent stimuli that trigger phasic dopamine release, which is associated with initiating goal-directed behaviors (Phillips *et al*, 2003; Roitman *et al*, 2004), enhance the signal to noise of phasic dopamine signaling and promote drug seeking.

Our finding that the net increase in cocaine-stimulated dopamine release is attenuated 24 h after salvA suggests neuroplasticity within mesolimbic circuits. Indeed, the expression of TH mRNA and protein, as well as a form of ERK-activated (phosphorylated) TH (pTH) is elevated in the VTA 24 h post-salvA. Furthermore, there is a significant increase in tissue levels of the dopamine metabolite DOPAC in the VTA as well as a strong trend for increased dopamine. Taken together, these results suggest that a compensatory neuroadaptation to KOR-mediated inhibition of dopamine release is upregulation of dopamine synthesis. Interestingly, salvA-induced TH mRNA in the VTA is associated with increased TH protein in the VTA but not the NAc, an afferent target of VTA dopamine neurons. There is precedent for this, as TH protein levels are increased in the VTA, but not the NAc, following 1 day of withdrawal from cocaine self-administration (Lu *et al*, 2003). Levels of pTH in the NAc, which most likely originate from dopaminergic terminals, are decreased 24 h post-salvA. Together with unchanged dopamine content in the NAc, these data suggest

that the delayed effects of KOR activation on dopamine synthesis occur in VTA dopaminergic neurons that project to regions other than the NAc (eg, prefrontal cortex). Although DAT mRNA levels are increased in the VTA 24 h after salvA, protein levels remain constant, which is consistent with the slow rate of *de novo* DAT protein synthesis (Kimmel *et al*, 2000).

An alternative, but not mutually exclusive, explanation for the delayed effect of salvA on cocaine-stimulated dopamine release is that KOR activation suppresses KOR signaling itself, which could indirectly promote dopamine release through loss of inhibitory input. Indeed, we found that prodynorphin, KOR, and *Gai2* mRNA levels are decreased in the NAc 24 h after salvA injection. Although not physiologically verified at this point, these findings are consistent with a large body of literature describing compensatory changes in neuropeptide and G-protein expression and function in response to manipulations of dopamine signaling within the mesolimbic system. Dopamine depletion results in decreased dynorphin expression (Gerfen, 2000), whereas the dopamine-releasing drug amphetamine stimulates transcription of dynorphin (Cole *et al*, 1995) and increases dynorphin release, which subsequently desensitizes KORs (Xia *et al*, 2008). The mechanism underlying KOR agonist-mediated KOR desensitization is not fully understood, but may involve the downregulation of G-proteins such as *Gai2*. We did not observe salvA-mediated changes in KOR-related gene expression in the VTA, suggesting that KORs to this area are unchanged, whereas dynorphin levels in NAc efferents to the VTA are decreased. The effect of decreased KOR expression on intrinsic NAc neurons is not well understood, in part because the phenotype of KOR-expressing medium spiny neurons is not known (Svingos *et al*, 2001). Consistent with desensitization of KOR signaling—regardless of mechanism—we found that the threshold-increasing effects of a second salvA injection were consistently (but not significantly) lower than those of the first salvA injection.

In subtle contrast to our findings, Ehrich *et al* (2014) showed that a 15-min pretreatment with the KOR agonist U50,488 attenuates, whereas a 1-h pretreatment potentiates, cocaine-induced conditioned place preferences (see also

McLaughlin *et al* (2006) and NAc dopamine release evoked by pedunculo-pontine tegmental nucleus stimulation. U50,488-mediated conditioned place aversions are observed at 15 min, but not 1 h, which seems to contradict the hypothesis that dysphoria enhances sensitivity to cocaine reward. However, we have shown previously that U50,488 produces immediate elevations in ICSS thresholds that last for at least 2 h (Russell *et al*, 2014). Although salvinorin and U50,488 have unique pharmacological properties (Liu-Chen, 2004), it is most likely that the observed differences in behavioral results between the current study and that of Ehrlich *et al* (2014) are due to the different aspects of reward-related behavior measured by place conditioning and ICSS (Carlezon, 2003; Carlezon and Chartoff, 2007). Unlike ICSS, place conditioning requires associative learning and memory between drug effect and context, which raises the possibility that an immediate effect of KOR activation is suppression of learning and memory processes. This is broadly consistent with the finding that KOR agonists inhibit long-term potentiation in the hippocampus (Terman *et al*, 1994) and produce deficits in attention (Nemeth *et al*, 2010).

The dynamic effects of KOR activation on affective state and neural circuits described here highlight the complexity of developing treatments for drug addiction and may help answer a long-standing divide in the literature. On one hand, numerous studies support the idea that KOR activation has drug-opposite effects and conclude that KOR agonists might have utility in combatting addiction (Mello and Negus, 2000; Shippenberg, 2009; Kivell *et al*, 2014). On the other hand, a growing body of evidence supports the idea that KOR activation has drug-withdrawal-like effects that promote addictive behavior and conclude that KOR antagonists should be effective in combatting addiction (Walker and Koob, 2007; Aldrich and McLaughlin, 2009; Bruchas *et al*, 2010; Jackson *et al*, 2010). This divide is well illustrated by a study in which the KOR antagonist JDTC blocks stress-induced reinstatement of cocaine seeking but potentiates cocaine-induced reinstatement (Beardsley *et al*, 2005). One possible resolution is the use of KOR partial agonists (Beguin *et al*, 2009), which would be expected to mute both the intoxicating effects and the withdrawal-induced depressive-like effects of drugs of abuse (Carlezon *et al*, 2009).

## FUNDING AND DISCLOSURE

The authors declare no conflict of interest.

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