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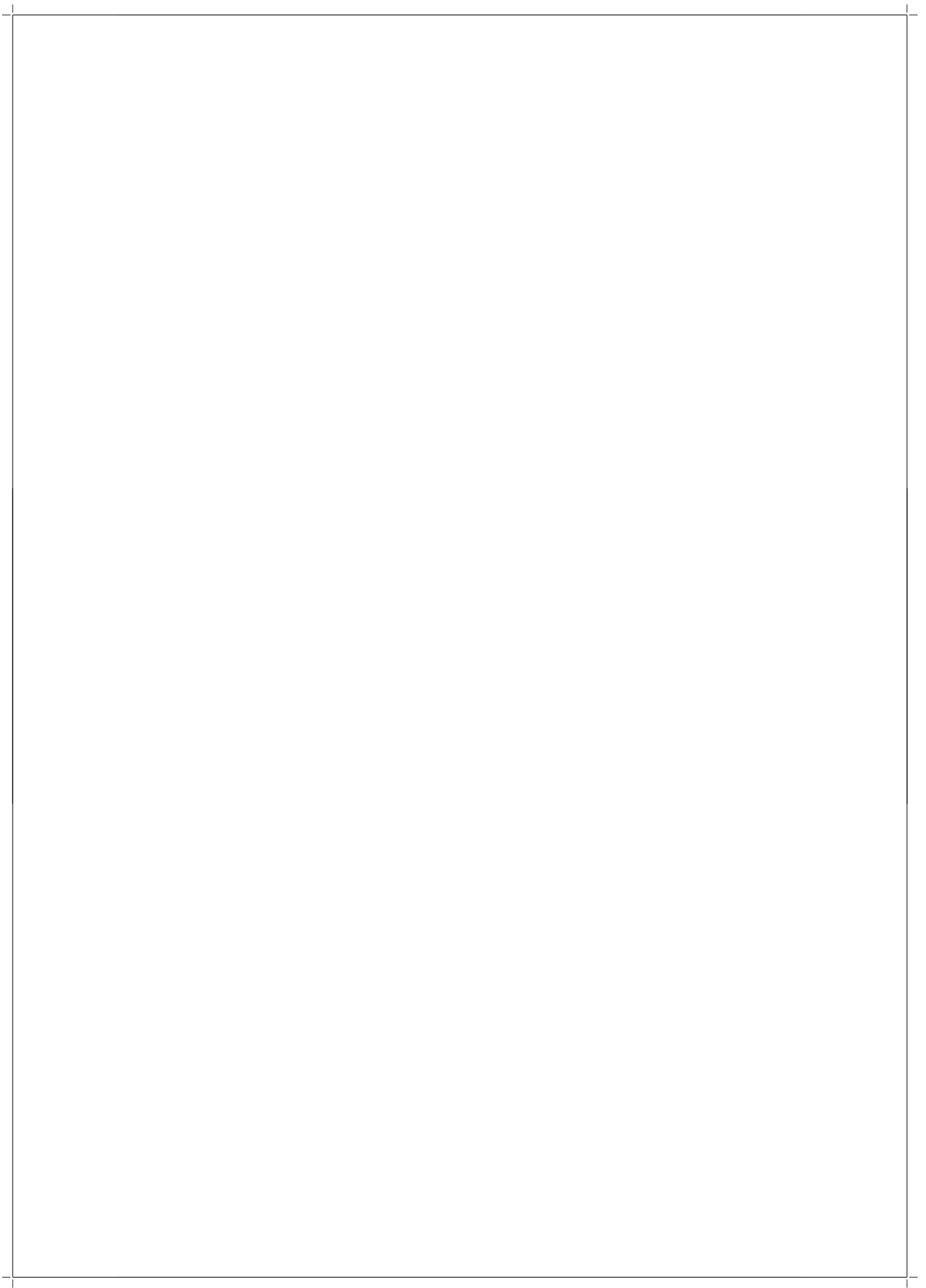
# Chapter 5

Relapse to heroin and sucrose seeking is associated with distinct patterns of in vivo endocannabinoid release in the rat medial prefrontal cortex

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# Chapter 5 Relapse-associated endocannabinoid release

## Abstract

It is well known that cannabinoid CB1 receptors, including those expressed in the medial prefrontal cortex (mPFC), play a pivotal role in mediating cue- and priming-induced relapse to drug and food seeking behavior. It remains to be determined, however, whether relapse behavior is actually associated with release of endocannabinoids (eCBs) such as 2-arachidonyl glycerol (2-AG) and anandamide. Thus, employing an operant rat model for relapse behavior, we first compared the effects of SR141716A (cannabinoid CB1 receptor antagonist), O-2050 (neutral cannabinoid CB1 receptor antagonist), and URB597 (inhibitor of the eCB catabolic enzyme fatty acid amide hydrolase (FAAH)) on relapse to heroin and sucrose seeking. Subsequently, a microdialysis technique was used to measure *in vivo* eCB release in the dorsal and ventral mPFC during heroin and sucrose seeking. Results demonstrated that SR141716A and O-2050, but not URB597, dose-dependently suppressed heroin and sucrose seeking. More importantly, microdialysis results indicated that both heroin and sucrose seeking were associated with enhanced mPFC 2-AG release. These changes in 2-AG release were more pronounced in heroin rats than in sucrose rats, and stronger in dorsal compared to ventral parts of the mPFC. In contrast, only heroin seeking was associated with decreased extracellular anandamide (AEA) and N-oleoylethanolamine (OEA) levels, specifically in the dorsal mPFC. Together, this is the first demonstration that relapse behavior is associated with altered *in vivo* eCB release in the mPFC and that this release is reinforcer-, analyte-, and brain region-specific.

## Introduction

A major challenge in treating heroin addiction, or any substance use-related disorder for that matter, is the persistent vulnerability to relapse when exposed to drug-associated cues, the previously abused drug (priming), or stressful situations, even after periods of protracted abstinence (De Wit and Stewart, 1981; Shaham et al., 2003; Koob and Volkow, 2010). The endogenous cannabinoid (eCB) system (Kano et al., 2009) is a neurotransmitter systems that is known to be critically involved in various addiction-related behaviors, and particularly relapse to drug and food seeking (Maldonado et al., 2006; Wiskerke et al., 2008; Justinova et al., 2009; Serrano and Parsons, 2011). A wealth of animal research clearly shows that blocking cannabinoid CB1 receptors by acute administration of the CB1 receptor antagonist SR141716A or its structural analogue AM251 attenuates cue- and priming-induced, but not stress-induced, reinstatement of drug and food seeking, whereas activating these receptors actually provokes reward seeking (De Vries et al., 2001; De Vries

and Schoffelmeer, 2005; Fattore et al., 2007; Serrano and Parsons, 2011; but see Vaughn et al., 2012). CB1 receptors located within the medial prefrontal cortex (mPFC) and nucleus accumbens appear to be particularly important in this respect (Kodas et al., 2007; Alvarez-Jaimes et al., 2008; Hiranita et al., 2008), in line with a pivotal role for prefrontal-accumbal projections in regulating this behavior (McFarland and Kalivas, 2001; McLaughlin and See, 2003; Van den Oever et al., 2010; Kalivas and Volkow, 2011). However, little is known about the underlying neurochemical substrates mediating these effects.

A fundamental question that remains unanswered is whether display of relapse behavior is indeed associated with *in vivo* release of eCBs including 2-arachidonyl glycerol (2-AG) and N-arachidonoyl-ethanolamide (anandamide, AEA), as has previously been observed for self-administration of several substances of abuse (Caille et al., 2007; Alvarez-Jaimes et al., 2009; Orio et al., 2009). Alternatively, given the putative inverse agonistic properties of SR141716A-like CB1 receptor antagonists (Pertwee, 2005), the relapse-preventing effects of such compounds might be due to reduction of constitutive activity rather than blockade of eCB-induced CB1 receptor activation. To resolve this issue, we compared the effects of systemic administration of SR141716A and the neutral CB1 receptor antagonist O-2050 on relapse to heroin and sucrose seeking. The effects of URB597 on these behaviors were also tested in view of recent reports that this inhibitor of fatty acid amide hydrolase (FAAH), the primary enzyme responsible for the hydrolysis of fatty acid ethanolamides including AEA and the non-cannabinoid N-oleoylethanolamine (OEA) (Cravatt et al., 1996), attenuated cue-induced reinstatement of nicotine and cocaine seeking, but not alcohol seeking (Cippitelli et al., 2008; Adamczyk et al., 2009; Forget et al., 2009). Subsequently, we employed an *in vivo* microdialysis technique to measure relapse-associated 2-AG, AEA, and OEA release in dorsal and ventral parts of the rat mPFC. These anatomically segregated mPFC subregions, comprising the anterior cingulate and dorsal prelimbic cortex and the ventral prelimbic and infralimbic cortex, respectively (Heidbreder and Groenewegen, 2003), may have distinct roles in relapse behavior (Peters et al., 2009; Van den Oever et al., 2010).

## Materials and Methods

*Subjects*
Male Wistar rats were obtained from Harlan CPB (Horst, The Netherlands). At the start of the experiments animals weighed approximately 250 grams, and were housed individually in macrolon cages (42.5 × 26.6 × 18.5 cm; length×width×height) under a reversed 12 hr light/dark cycle (lights on at 7.00 p.m.) at controlled room temperature (21 ± 2 °C) and relative humidity of 60 ± 15%. Throughout the entire experiment, animals had *ad libitum* access to water and standard rodent food pellets (Harlan Teklad Global Diet, Blackthorn, UK). All experiments were conducted with the approval of the animal ethical committee of the VU University, Amsterdam, The Netherlands.

*Drugs*

SR141716A was synthesized and kindly provided by Abbott Laboratories (Weesp, The Netherlands), O-2050 was purchased from Tocris Bioscience (Bristol, UK), and URB597 was purchased from Sigma Aldrich (St. Louis, MO, USA). All drugs were dissolved in vehicle (ethanol:tween80:saline, ratio 1:1:18). Chromatographic standards AEA, 1(3)-arachidonoylglycerol (1-AG), 2-AG, and (S)-(+)-arachidonyl-2'-hydroxy-1'-propylamide (S-2 methanandamide) were purchased from Cayman Chemical (Ann Arbor, MI, USA). For systemic pharmacology experiments, drugs were freshly prepared on the test day, and administered intraperitoneally (i.p.) in a volume of 1 ml/kg bodyweight according to a Latin square within-subjects design. Drug doses and injection times were based on previous studies in our laboratories (Chapter 3; De Vries et al., 2001; Wiskerke et al., 2012), such that URB597 was injected 60 min prior to testing, whereas SR141716A and O-2050 were injected 30 min prior to testing.

*Surgery*

All surgeries were performed prior to the start of the self-administration phase. For experiments involving heroin self-administration, rats were surgically implanted with intravenous silicon catheters (outer diameter: 0.6 mm, inner diameter: 0.3 mm) in the right jugular vein under gas anesthesia (1.5-2% isoflurane). The catheter was secured to the vein with two sutures and passed subcutaneously to the top of the skull. The distal end of the catheter was attached to a connector pedestal (Plastics One, Dusseldorf, Germany) anchored to the skull using four surgical screws and dental cement. Post-surgery, rats received 0.5 ml/kg of the analgesic Ketofen (1%; Merial, Amstelveen, The Netherlands) and 0.3 ml/kg of the antibiotic Baytril (2.5%; Bayer, Mijdrecht, The Netherlands). Animals had at least 7 days to recover after the surgery. Throughout the remainder of the experiment, catheter patency was maintained by daily infusion of a 0.1 ml sterile saline solution containing heparin (47.5 IU/ml) and the antibiotic gentamicin (0.08 mg/ml).

For experiments involving *in vivo* microdialysis, animals were anaesthetized using 1.5-2% isoflurane vapors and mounted on a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) for implantation of a unilateral microdialysis guide cannula (Plastics One, Roanoke, VA, USA) aimed to end above either one of two target regions, the dorsal or ventral part of the medial prefrontal cortex (mPFC), using the following coordinates (in mm) from bregma (calculated from Paxinos and Watson, 1998): dorsal mPFC: anteroposterior (AP) +3.00; mediolateral (ML) +0.8 mm; and dorsoventral (DV) -1.90 mm from dura; ventral mPFC: AP +3.00; ML +1.0 mm; and DV -3.60 mm from dura. Guide cannulae were inserted under a sagittal angle (5° angle relative to the midline sagittal plane of the skull) to prevent damage to blood vessels on top of the mPFC. Probe placements within each test group were randomized between both hemispheres. For heroin animals, the cannulation surgery occurred at the time of i.v. catheter implantation, and the guide cannula was anchored to the skull together with the connector pedestal of the i.v. catheter using four stainless steel screws and dental acrylic cement. For sucrose animals, the guide cannula was anchored to the skull together with a stainless screw instead of a connector pedestal, in order to

connect these animals to a liquid swivel during self-administration sessions (for reasons of heroin-sucrose comparison as well as habituation to the microdialysis procedures).

*Apparatus*
Self-administration of heroin and sucrose was conducted in 32 identical operant chambers (Med Associates Inc., St. Albans, VT, USA) in sound-attenuating ventilated cubicles. On one wall, two differentially shaped levers were situated, of which one was a retractable, active lever, which was presented during a session. The other lever, the inactive lever, was non-retractable. Yellow cue lights were situated above both levers. Furthermore, all boxes were equipped with a dim red house light and an auditory clicker (ENV-135M, Med Associates Inc.) in the opposite wall. In heroin self-administration experiments, rats were connected to an infusion pump (PHM-100, Med Associates Inc.) via a liquid swivel for i.v. delivery of heroin. During sucrose self-administration on the other hand, sucrose was delivered to a liquid receptacle positioned in the wall in between the two levers.

*Heroin- and sucrose self-administration procedure*
The rats were trained to self-administer heroin (diacetylmorphine-HCl, dissolved in 0.9% sterile saline, Slotervaart Hospital, The Netherlands) or 10% sucrose solution by pressing the active lever on a fixed ratio (FR) 1 schedule of reinforcement, i.e. every lever press was reinforced with 100 µg/kg heroin (infused i.v. in a volume of ~42 µl) or 190 µl sucrose solution (in a liquid receptacle), delivered over 2 or 4.2 s, respectively. Rats were trained Monday through Friday during twice daily 1 h (sucrose) or 2 h (heroin) sessions, separated by 3 h. Reinforcer delivery was accompanied by a 5 s presentation of the cue light situated above the active lever in combination with 1 Hz auditory clicks. Upon each drug infusion, a time-out period of 15 s was introduced, during which lever pressing had no consequences. Responses on the inactive lever never had programmed consequences. Responses on the active and inactive lever were registered, during availability of the drug as well as during the time-out period. A house light was turned on during the entire session. After 14 sessions, the schedule of reinforcement was increased to FR2 (sessions 15-18) and FR4 (sessions 19-30), meaning that respectively every second or fourth active lever press was reinforced. Following the final self-administration session, rats remained in the homecage and abstained from drugs until relapse testing.

*Post-abstinence relapse to heroin and sucrose seeking*
On a relapse test day, rats were tested under conditions identical to those of a self-administration session, with the important exception that no heroin or sucrose reinforcers could be obtained. In microdialysis experiments, rats were only subjected to a relapse test once, following an abstinence period of three to five weeks, whereas for systemic pharmacology experiments relapse tests were conducted once every week starting on abstinence day 21.

*In vivo microdialysis*
On the morning of the microdialysis experiment, animals were briefly anesthetized with isoflurane, and a microdialysis probe with 1mm active length polyethyl sulfone dialysis membrane and 15kDa MW cutoff (MAB 6.20.1; Microbiotech/se AB, Stockholm, Sweden) was inserted and secured to the previously implanted guide cannula using epoxy. Following implantation, the microdialysis probe was perfused with freshly prepared artificial CSF (aCSF) composed of 145mM NaCl, 2.8mM KCl, 1.2mM MgCl2, 1.2mM CaCl2, 5.4mM D-Glucose, 0.25mM ascorbic acid (pH 7.2–7.4) at a low flow rate (0.3 μl/min) to allow for the re-equilibration of neurotransmitter levels.

Approximately 5 h later, and 60-90 min prior to initiation of sample collection (which always started around 1.00 p.m.), the aCSF solution was replaced with an aCSF solution containing 30% (w/v) (2-hydroxypropyl)-β-cyclodextrin (Sigma Aldrich) and the flow rate was increased to 0.6 μl/min. As previously described (e.g. Caille et al., 2007), inclusion of hydroxypropyl-β-cyclodextrin substantially increases the recovery of endocannabinoids (eCBs) by microdialysis. From the initiation of sample collection until the end of the experiment, dialysis samples were collected every 10 min, and stored on dry ice during the experiment, and then at -80°C until analysis for eCB content using liquid chromatography coupled with mass spectrometry. For all rats, 6 samples were collected in the homecage as a pre-relapse baseline. Subsequently, rats were transferred to an operant chamber and subjected to a 60 min cue+context-induced relapse test. Following the relapse test, rats were transferred back to the homecage where 6 additional samples were collected as a post-relapse measurement.

*Liquid chromatography/mass spectrometry analysis of dialysate endocannabinoid content*
Sample analysis of AEA, OEA, 2-AG and 1-AG was performed essentially as previously described (e.g. Caille et al., 2007) using liquid chromatography coupled with mass spectrometry. Briefly, 5 μl of microdialysate was mixed with 5 μl of 25 nM S-2 methanandamide internal standard and subsequently loaded onto a pre-column (1 x 10 mm, Haisil HL C18 5 μm, Higgins Analytical Inc., Mountain View, CA, USA). The loaded pre-column was washed for 4 min using 10% MeOH (v/v) mobile phase delivered at 53 μl/min to remove hydrophilic species and especially the hydroxypropyl-β-cyclodextrin from the sample. Next, mobile phase flow through the pre-column was reversed using a switch valve, and analytes were washed off the pre-column and delivered to the analytical column (0.5 x 150 mm, Haisil HL C18 3 μm; Higgins Analytical Inc.) using 80% MeOH (v/v) isocratic mobile phase delivered at 9 μl/min. The analytical column was connected to an 1100 LC system that was coupled to an 1946D mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) that was run in positive Selected Ion Monitoring mode to enhance detection of low-abundance eCB, hence Mass/charge ratios used were: AEA (370.3 (M+ 1Na), OEA (348.2 (M+ 1Na), 2-AG and 1-AG (401.3 (M+ 1Na), and S-2 methanandamide (384.3 (M+ 1Na). Quantification was achieved using daily generated external calibration curves constructed from three standard concentrations (each in duplicate). Under these conditions, the limit of detection was ~0.1nM for all analytes.

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*Histology*
Following completion of the behavioral procedures, animals used in the *in vivo* microdialysis experiments were deeply anaesthetized using sodium pentobarbital (Ceva Sante Animale BV, Maassluis, The Netherlands; 60 mg/ml, i.p.). Subsequently, animals were perfused transcardially with 100 ml 0.9% NaCl, followed by 500 ml 4% paraformaldehyde in 0.1M phosphate buffered saline (PBS, pH 7.2). Brains were removed rapidly and post-fixed for 1 h in the same fixative at room temperature, then stored in 10% sucrose in 0.1M PBS at 4 °C. Coronal sections of 35 µm were cut on a cryostat and subsequently stained with thionine for determination of the probe implantation sites. Only animals with correct probe placements were included in the analyses.

*Statistical analyses*
All data were analyzed using the Statistical Package for the Social Sciences version 15.0 (SPSS, Chicago, IL, USA). The level of probability for statistically significant effects was set at 0.05. All graphs were produced using GraphPad Prism version 5.02 for Windows (GraphPad Software, San Diego, CA, USA).

Behavioral data were subjected to repeated measures analysis of variance (ANOVA). For systemic pharmacology experiments, drug dose was used as a within-subjects variable. In microdialysis experiments, session number (self-administration phase) or time bin (relapse testing) was used as within-subjects variable, whereas reinforcer (heroin or sucrose) and/ or brain region (dmPFC or vmPFC) were used as between-subjects factors. Data were checked for normal distribution using Kolmogorov-Smirnov tests, whereas homogeneity of variance across groups was determined using Mauchly's tests for equal variances. In case of violation of homogeneity, Huynh-Feldt epsilon (ε) adjusted probability values and therefore more conservative probability values were depicted and used for subsequent analyses. In case of statistically significant main effects, further post hoc comparisons were conducted using Bonferroni multiple comparison tests. Because rats in the systemic pharmacology experiments were subjected to three relapse tests during which behavior was no longer reinforced by drug delivery, we observed some extinction of reward seeking

**Table 5.1.** Mean baseline medial prefrontal cortex (mPFC) dialysate 2-AG, AEA, and OEA concentrations (± SEM) per test group.

| Brain region | Reinforcer | n | 2-AG (nM) | AEA (nM) | OEA (nM) |
|---|---|---|---|---|---|
| Dorsal mPFC | Heroin | 13 | 6.74 ± 0.75 | 0.80 ± 0.09 | 14.40 ± 2.10 |
| | Sucrose | 10 | 5.59 ± 0.69 | 0.71 ± 0.13 | 12.97 ± 3.27 |
| Ventral mPFC | Heroin | 13 | 6.03 ± 0.72 | 0.82 ± 0.10 | 16.26 ± 2.93 |
| | Sucrose | 10 | 5.30 ± 0.60 | 0.88 ± 0.09 | 9.19 ± 1.32 |

behavior over the course of the three relapse tests. Particularly in the sucrose experiments, this confounded the results and disturbed the normal distribution of the data. Hence, for statistical analyses (uncorrected data are displayed in graphs), we normalized all data by calculating correction factors based on a comparison between the responses made under vehicle conditions on day 1 versus respectively day 2 and day 3.
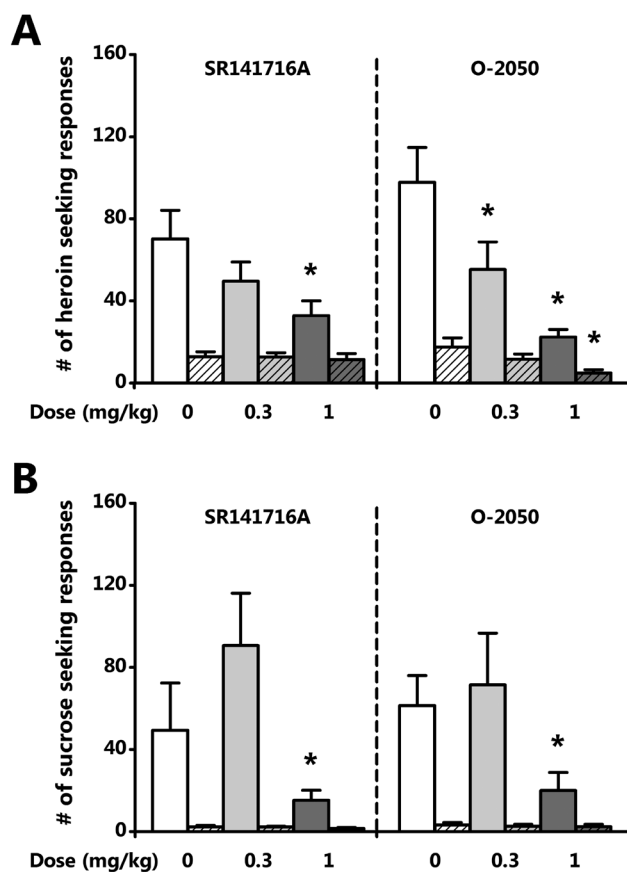
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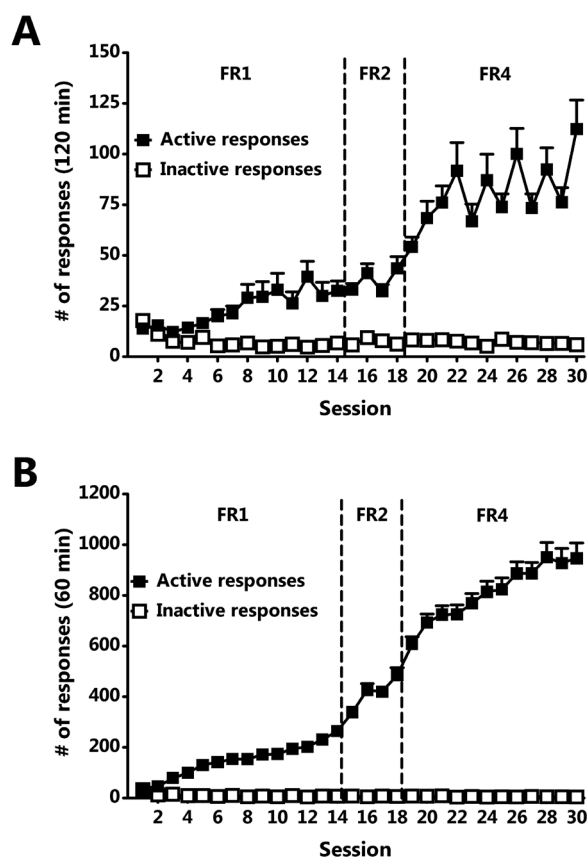


**Figure 5.1. Acquisition of heroin and sucrose self-administration.** Shown are the mean (± SEM) number of active and inactive lever presses (including time out responses) made by heroin (A) and sucrose (B) self-administering rats in twice daily sessions. Heroin and sucrose sessions lasted for 60 or 120 min, respectively. Over the course of the self-administration period, the reinforcement schedule increased from fixed ratio (FR) 1 to FR4. In total, data from $n$ = 56 heroin rats and $n$ = 43 sucrose rats, used in either the systemic pharmacology or microdialysis experiments, is depicted.

**B**



For microdialysis data, group differences in baseline dialysate 2-AG, AEA, and OEA concentrations were evaluated using two-way ANOVAs with reinforcer type and brain region as between-subjects factors. Importantly, as shown in Table 5.1, no differences in baseline dialysate content were found between any of the four experimental groups (brain region effect: $F_{1,41}$ = 0.12, NS; drug effect: $F_{1,41}$ = 3.23, NS; brain region x drug x analyte effect: $F_{2,82}$ = 1.66, NS, $\varepsilon$ = 0.60). Subsequently, per test group, dialysate 2-AG, AEA, and OEA levels were transformed to percentages of mean baseline dialysate concentration (set at 100%) for evaluation of changes in dialysate content during and following the relapse test as performed by repeated measures ANOVAs, using timebin and analyte as within-

subjects variables and reinforcer and probe placement as between-subjects factors. In case of significant overall effects, Bonferroni multiple comparison tests were used for post hoc comparisons. In addition, AUC measures for 2-AG, AEA, and OEA were calculated for each animal by subtracting 100 from each percentage of baseline data point and summing all data points collected from the onset of the relapse test on (t = 0-120 min). AUC data was analyzed using Student's T-tests. Finally, Pearson's correlation analyses were used to test whether observed changes in 2-AG, AEA, and OEA dialysate levels (AUC data) correlated with the displayed relapse behavior (total active responses made).

## Results

To be able to study the role of the eCB system, and particularly eCBs in the mPFC, in relapse to heroin and sucrose seeking in more detail, rats first had to learn to self-administer heroin or sucrose during twice daily 1 h (sucrose) or 2 h (heroin) sessions.



**Figure 5.2. The cannabinoid CB1 receptor antagonists SR141716A and O-2050 suppress relapse to heroin and sucrose seeking.** The effects of systemic administration of SR141761A and O-2050 on the mean (± SEM) number of active (clear bars) and inactive (striped bars) lever presses (including time out responses) made during a 30 min relapse to heroin seeking (A) or sucrose seeking (B) test. For each compound, $n$ = 7-10 rats were included in each analysis. Drug doses are expressed as mg/kg. * $p < 0.05$ versus respective vehicle.

As illustrated in Figure 5.1, rats included in this study acquired drug self-administration, as reflected by a clear discrimination between the active and inactive levers over increasing FR schedules of reinforcement (FR1 - FR4). Moreover, following initial acquisition (FR1 sessions), rats displayed a stable pattern of heroin or sucrose intake over the remaining sessions. Over the course of an experiment, a heroin-trained rat on average earned 309.82 ± 18.38 heroin rewards (a total of 30.98 ± 1.84 mg/kg heroin, average of 1.03 ± 0.06 mg/kg per session), whereas a sucrose-trained rat on average obtained 2405.63 ± 56.14 sucrose rewards (a total of 457.07 ± 10.67 mL 10% sucrose solution, average of 15.24 ± 0.36 mL per session). In line with previous reports (Schmidt et al., 2005; Koya et al., 2006), despite the different number of cue-reward pairings that heroin and sucrose rats received during self-administration, heroin and sucrose rats displayed comparable levels of reward seeking during a relapse test (see below).

*The cannabinoid CB1 receptor antagonists SR141716A and O-2050, but not the fatty acid amide hydrolase inhibitor URB597, suppress relapse to heroin and sucrose seeking*
As a first step in studying the role of the eCB system in relapse to heroin and sucrose seeking, we evaluated whether the inhibitory effects of the CB1 receptor antagonist SR141716A on these behaviors (De Vries et al., 2003, 2005; Alvarez-Jaimes et al., 2008) result from the putative inverse agonistic properties of this compound. Thus, using four cohorts of rats, we compared the effects of systemic administration of SR141716A and the neutral CB1 receptor antagonist O-2050 (Canals and Milligan, 2008; Wiley et al., 2010) on relapse to heroin and sucrose seeking following a period of drug abstinence. Results showed that pretreatment with either SR141716A or O-2050 dose-dependently suppressed conditioned heroin seeking (Figure 5.2a), as reflected by a treatment effect on total number of active responses made during a relapse test (SR141716A: $F_{2,18}$ = 6.82, $p$ = 0.006; O-2050: $F_{2,18}$ =
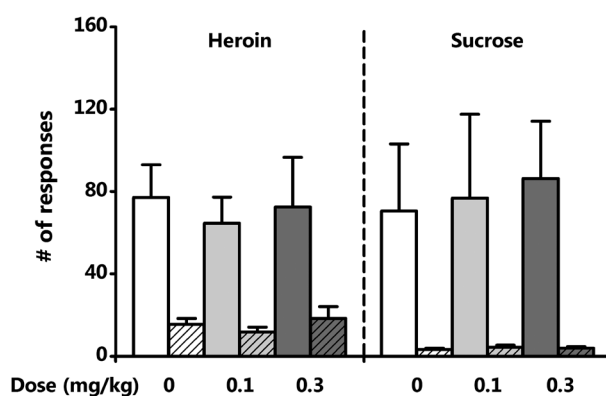


**Figure 5.3. The fatty acid amide hydrolase inhibitor URB597 does not affect relapse to heroin or sucrose seeking**. The effects of systemic administration of URB597 on the mean (± SEM) number of active (clear bars) and inactive (striped bars) lever presses (including time out responses) made during a 30 min relapse to heroin or sucrose seeking test. For each compound, $n$ = 8-10 rats were included in each analysis. Drug doses are expressed as mg/kg.
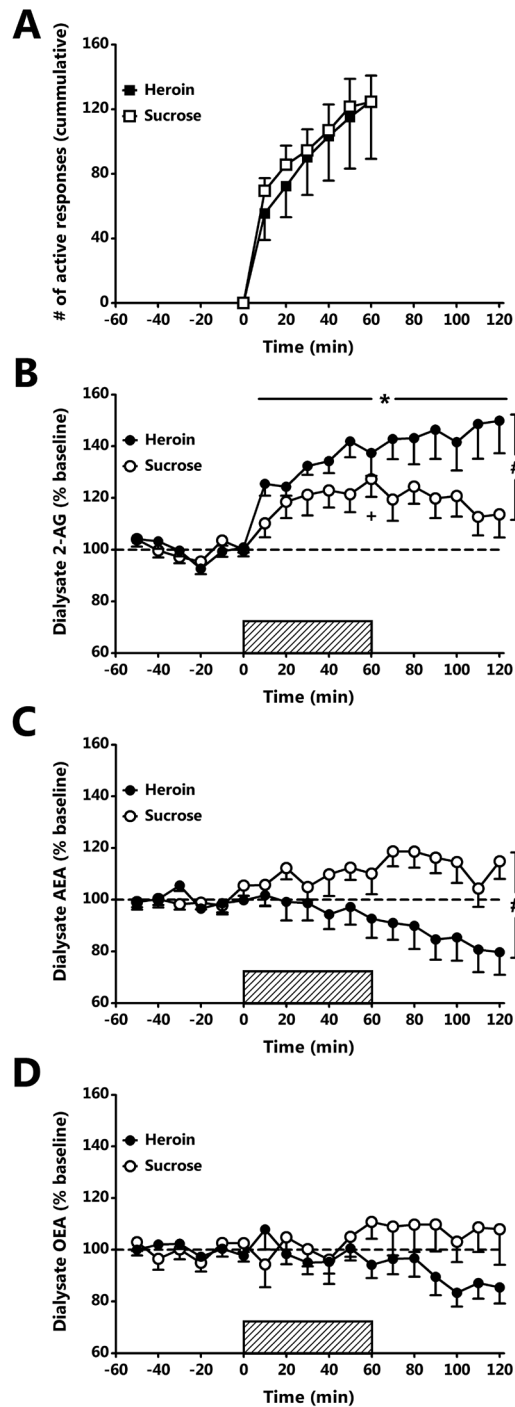
**Figure 5.4. Relapse to heroin and sucrose seeking are associated with distinct patterns of endocannabinoid release in the dorsal part of the medial prefrontal cortex.** Shown in A are the active lever presses (including time out responses) made by rats during a 60 min (start at $t = 0$ min) test for relapse to heroin or sucrose seeking. Simultaneously measured changes in extracellular levels of 2-AG, AEA, and OEA in the dmPFC are shown in B-D. In the latter figures the shaded area from $t = 0$-60 min indicates the time rats were in the operant chamber being subjected to a relapse test. Data depict mean (± SEM) per 10 min bin. In total, $n = 12$-13 and $n = 10$ rats were included in the analyses for heroin and sucrose, respectively. * and $^+$ $p < 0.05$ versus mean baseline dialysate values in heroin and sucrose rats, respectively; # $p < 0.05$ compared to sucrose rats.

10.59, $p$ = 0.007, $\varepsilon$ = 0.57). Post hoc analyses showed that 1 mg/kg SR141716A and 0.3 and 1 mg/kg O-2050 decreased active responding as compared to vehicle. The highest dose of O-2050 also suppressed responding on the inactive lever ($F_{2,18}$ = 5.89, $p$ = 0.01), however, a two-way repeated measures ANOVA showed that this effect was less pronounced than the effects of O-2050 on active lever responding (lever x treatment effect: $F_{2,18}$ = 8.55, $p$ = 0.01, $\varepsilon$ = 0.63). SR141716A treatment did not affect inactive responding ($F_{2,18}$ = 0.60, NS). Both SR141716A and O-2050 pretreatment also suppressed relapse to sucrose seeking (Figure 5.2b; SR141716A: $F_{2,12}$ = 7.27, $p$ = 0.009; O-2050: $F_{2,14}$ = 6.06, $p$ = 0.01), though this effect required higher antagonist doses than necessary for significant reduction of heroin seeking. Neither compound influenced responding on the inactive lever during the sucrose seeking tests (SR141716A: $F_{2,12}$ = 1.29, NS, $\varepsilon$ = 0.57; O-2050: $F_{2,14}$ = 0.78, NS). These results indicate that the relapse-suppressing effects of SR141716A are not due to inverse agonism, but rather result from blockade of eCB-induced CB1 receptor activation.

To elucidate the eCBs that mediate relapse to heroin and sucrose seeking, we subsequently explored the effects of systemic inhibition of eCB catabolism on these behaviors. The primary eCBs in the brain, 2-AG and AEA, are hydrolyzed by the enzymes monoacylglycerol lipase (MAGL) and FAAH, respectively (Cravatt et al., 1996; Dinh et al., 2002). Because potent and selective MAGL inhibitors for rat research are to date not available, we decided to study the effects of systemic administration of the FAAH inhibitor URB597. URB597 was previously reported to attenuate cue-induced reinstatement of nicotine and cocaine seeking, but not alcohol seeking (Cippitelli et al., 2008; Adamczyk et al., 2009; Forget et al., 2009). The current results (Figure 5.3) extend these findings by indicating that URB597 treatment did not affect conditioned heroin seeking (active responding: $F_{2,18}$ = 0.38, NS; inactive responding: $F_{2,18}$ = 1.14, NS) or conditioned sucrose seeking (active responding: $F_{2,14}$ = 1.97, NS; inactive responding: $F_{2,14}$ = 0.47, NS). Together, these data suggest that AEA may not be the primary eCB mediating acute relapse behavior in rats.

*Relapse to heroin and sucrose seeking are associated with distinct patterns of endocannabinoid release in the dorsal part of the medial prefrontal cortex*
As a more direct way to study the role of eCBs in heroin and sucrose seeking, we next performed a series of microdialysis experiments to measure *in vivo* release of 2-AG and AEA as well as the non-CB1 receptor-targeting FAAH substrate OEA during a test for relapse to heroin or sucrose seeking (see Table 5.1 for untransformed baseline dialysate contents). Studies by our and other laboratories have reported an important role for the dorsal part of the mPFC (dmPFC) in cue-induced reinstatement of heroin seeking (Schmidt et al., 2005; LaLumiere and Kalivas, 2008; Rogers et al., 2008). Hence, relapse-associated alterations in 2-AG, AEA, and OEA release were first studied in the dmPFC. Results showed that re-introduction to the drug self-administration context and associated cues following a period of abstinence caused relapse to heroin and sucrose seeking in rats implanted with a microdialysis probe in the dmPFC (Figure 5.4a), with both heroin and sucrose trained rats clearly discriminating between the active and inactive lever (inactive lever press data not shown) and displaying a similar pattern of active responding over time (reinforcer effect:

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$F_{1,21}$ = 0.049, NS; reinforcer x timebin effect: $F_{5,105}$ = 0.28, NS, $\varepsilon$ = 0.32). Subsequent analyses of the microdialysis results showed distinct, analyte-specific alterations in dialysate content over time in heroin- and sucrose-trained rats (reinforcer effect: $F_{1,20}$ = 0.97, NS; analyte effect: $F_{2,40}$ = 14.70, $p$ < 0.001; reinforcer x analyte x timebin effect: $F_{24,480}$ = 2.41, $p$ < 0.001). Detailed analyses per analyte showed that both conditioned heroin and sucrose seeking were associated with increased 2-AG release in the dmPFC (Figure 5.4b; heroin: $F_{12,144}$ = 5.22, $p$ < 0.001; sucrose: $F_{12,108}$ = 1.87, $p$ = 0.05). However, the effects were more pronounced and persistent in the heroin rats as compared to sucrose rats (reinforcer effect: $F_{1,21}$ = 4.69, $p$ = 0.04). Post hoc test results supported this conclusion, since extracellular 2-AG levels in heroin rats were significantly enhanced as compared to baseline from the onset of the relapse test onwards until the end of the experiment ($t$ = 10-120 min), whereas in sucrose rats 2-AG release significantly deviated from baseline only at the end of the relapse test ($t$ = 60 min). Relapse to heroin seeking was also associated with overall effects on extracellular AEA and OEA levels (decline) in the dmPFC (Figure 5.4c,d; AEA: $F_{12,132}$ = 2.29, $p$ = 0.01; OEA: $F_{12,144}$ = 2.68, $p$ = 0.003), although post hoc tests revealed no specific point in time where levels of either analyte significantly deviated from baseline. It became evident from additional separate analyses performed over dialysate samples collected during vs. following the relapse test that the heroin seeking-associated decline in AEA
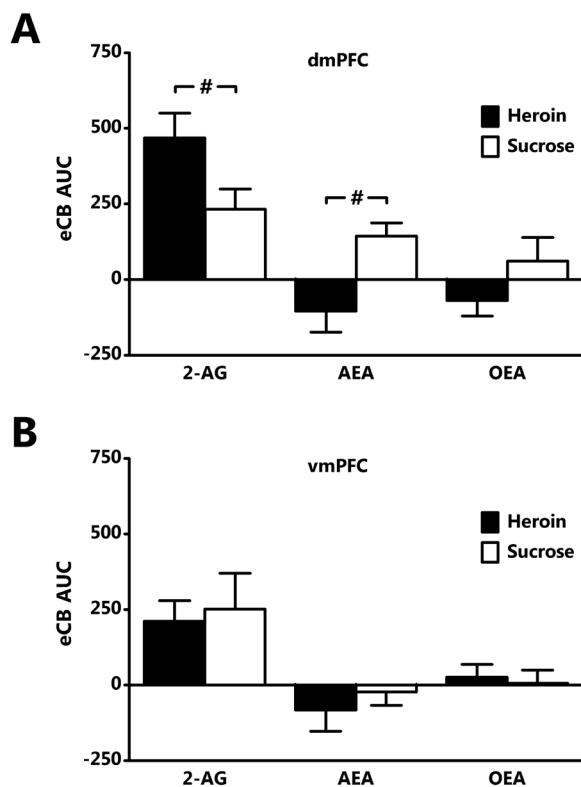


**Figure 5.5. Comparison of alterations in medial prefrontal cortex dialysate levels of 2-AG, AEA, and OEA observed during relapse to heroin and sucrose seeking.** Shown are the Area Under the Curve (AUC) data summarizing the observed changes in extracellular 2-AG, AEA, and OEA levels in the dorsal (A) and ventral (B) parts of the mPFC of heroin and sucrose rats during and following a relapse test ($t$ = 0-120 min). Each bar represents the mean ± SEM of data from $n$ = 10-13 rats. # $p$ < 0.05 compared to sucrose rats.

and OEA release occurred solely during the post-session period of dialysate sampling and did not occur during elicitation of heroin seeking behavior (relapse AEA: $F_{6,66}$ = 0.56, NS; relapse OEA: $F_{6,72}$ = 1.35, NS; post-relapse AEA: $F_{6,66}$ = 2.47, $p$ = 0.033; post-relapse OEA: $F_{6,72}$ = 3.22, $p$ = 0.007). In contrast, relapse to sucrose seeking was not associated with changes in dmPFC AEA or OEA release (Figure 5.4c,d; AEA: $F_{12,108}$ = 1.01, NS; OEA: $F_{12,108}$ = 0.60, NS). Consequently, a reinforcer effect (heroin vs. sucrose) was found for changes in dmPFC AEA but not OEA levels (AEA: $F_{1,20}$ = 8.35, $p$ = 0.009; OEA: $F_{1,21}$ = 2.20, NS). The distinct effects of heroin vs. sucrose seeking are further exemplified by comparisons of Area Under the Curve (AUC) data (Figure 5.5a) that demonstrate significantly greater elevations in dialysate 2-AG during and after heroin vs. sucrose seeking (2-AG: $t_{21}$ = -2.16, $p$ = 0.04) and opposite effects of heroin vs. sucrose seeking on AEA release (AEA: $t_{20}$ = 2.89, $p$ = 0.009; OEA: $t_{21}$ = 1.48, NS). Finally, Pearson's correlation analyses on the obtained behavioral and neurochemical data from individual animals did not indicate any significant correlations between reward seeking behavior and any neurochemical changes, nor between the different neurochemical effects, for either heroin or sucrose rats (data not shown).

*Relapse to heroin and sucrose seeking are associated with a subtle increase in extracellular 2-AG levels in the ventral part of the medial prefrontal cortex*
Recently, the ventral part of the mPFC (vmPFC) has also been implicated in cue- and/or context-induced reinstatement of heroin seeking by our and other laboratories (Ovari and Leri, 2008; Rogers et al., 2008; Van den Oever et al., 2008; Bossert et al., 2011). Therefore, relapse-associated alterations in 2-AG, AEA, and OEA release in the vmPFC were studied as well. Results showed that both heroin- and sucrose-trained rats implanted with a microdialysis probe in the vmPFC (Figure 5.6a) displayed robust reward seeking behavior upon re-introduction to the self-administration context and associated cues following a period of abstinence. Both groups of rats clearly discriminated between the active and inactive lever (inactive lever press data not shown) and displayed a similar pattern of active responding over time (reinforcer effect: $F_{1,21}$ = 2.01, NS; reinforcer x timebin effect: $F_{5,105}$ = 0.68, NS, $\varepsilon$ = 0.38). Importantly, an additional two-way ANOVA over repeated measures showed that relapse behavior displayed by heroin- and sucrose-trained, vmPFC-implanted rats did also not differ from that displayed by dmPFC-implanted rats (brain region effect: $F_{1,42}$ = 0.23, NS; brain region x reinforcer x timebin effect: $F_{5,210}$ = 0.63, NS, $\varepsilon$ = 0.36). In contrast, although heroin and sucrose seeking produced similar analyte-specific patterns of lipid alterations in both parts of the mPFC, a significant effect of brain region (dmPFC vs. vmPFC) was evident (brain region effect: $F_{1,41}$ = 1.73, NS; brain region x reinforcer x analyte x timebin effect: $F_{24,984}$ = 2.47, $p$ = 0.006, $\varepsilon$ = 0.43). In specific regard to the vmPFC, there was a similar, analyte-specific pattern of alterations in dialysate content in heroin- and sucrose-trained rats (reinforcer effect: $F_{1,21}$ = 0.17, NS; analyte effect: $F_{2,42}$ = 12.37, $p$ < 0.001; reinforcer x analyte x timebin effect: $F_{24,504}$ = 1.09, NS). Detailed analyses per analyte showed comparable subtle increments in vmPFC 2-AG release during both conditioned heroin and sucrose seeking (Figure 5.6b; heroin: $F_{12,144}$ = 2.73, $p$ = 0.002; sucrose: $F_{12,108}$ = 2.59, $p$ = 0.005). Post hoc tests indicated that extracellular 2-AG levels in heroin rats were significantly enhanced as compared to baseline at the start of the relapse test and
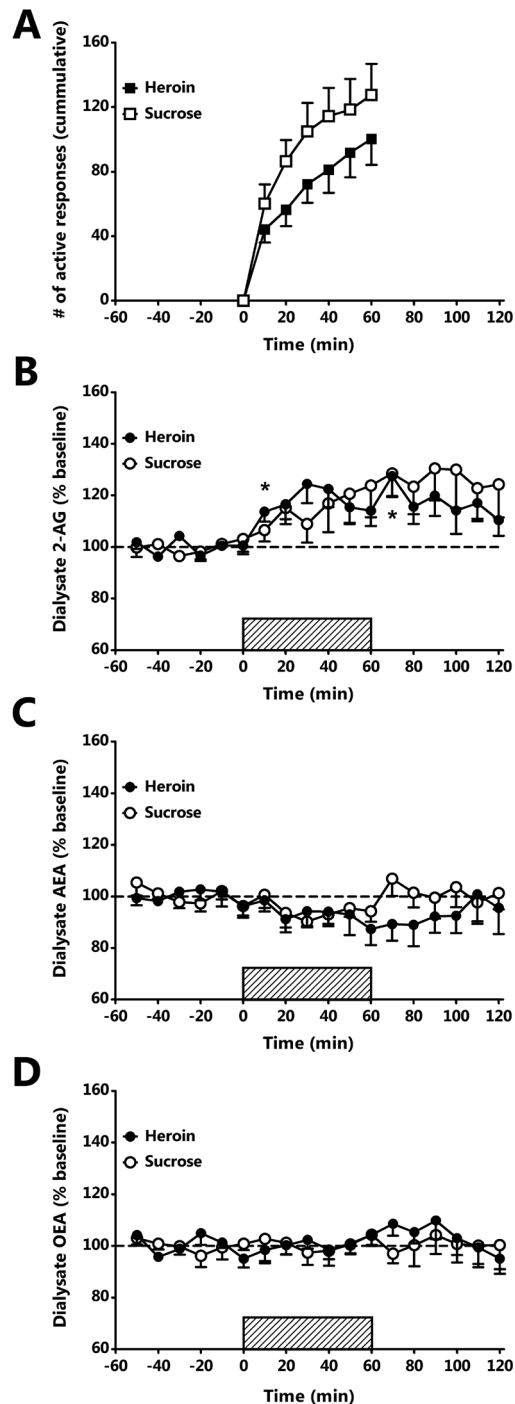
**Figure 5.6. Relapse to heroin and sucrose seeking are associated with similar, subtle changes in endocannabinoid release in the ventral part of the medial prefrontal cortex.** Shown in A are the active lever presses (including time out responses) made by rats during a 60 min (start at $t$ = 0 min) test for relapse to heroin or sucrose seeking. Simultaneously measured changes in extracellular levels of 2-AG, AEA, and OEA in the vmPFC are shown in B-D. In the latter figures the shaded area from $t$ = 0-60 min indicates the time rats were in the operant chamber being subjected to a relapse test. Data depict mean (± SEM) per 10 min bin. In total, $n$ = 13 and $n$ = 10 rats were included in the analyses for heroin and sucrose, respectively. * $p$ < 0.05 versus mean baseline dialysate values in heroin rats.

immediately following the end of the test ($t$ = 10 and 70 min), whereas in sucrose rats 2-AG release never significantly deviated from baseline. However, while sucrose seeking produced similar 2-AG elevations in both the dmPFC and vmPFC (brain region effect: $F_{1,18}$ = 0.02, NS; brain region x time effect: $F_{12,216}$ = 0.93, NS), the effect of heroin seeking on 2-AG was less pronounced in the vmPFC vs. dmPFC (brain region: $F_{1,24}$ = 5.86, $p$ = 0.023; brain region x time effect: $F_{12,288}$ = 2.52, $p$ = 0.004). These effects resulted in a significant interaction effect between brain region and reinforcer type (brain region x reinforcer x timebin effect: $F_{12,504}$ = 2.60, $p$ = 0.002). In further contrast to the dmPFC, no significant changes in vmPFC dialysate AEA or OEA levels were evident as a consequence of either heroin or sucrose seeking (Figure 5.6c,d; heroin AEA: $F_{12,144}$ = 0.81, NS; heroin OEA: $F_{12,144}$ = 1.33, NS; sucrose AEA: $F_{12,108}$ = 1.02, NS; sucrose OEA: $F_{12,108}$ = 0.15, NS). Comparisons of AUC data (Figure 5.5b) underscored the conclusion that heroin and sucrose seeking produced comparable subtle increases in vmPFC 2-AG levels (2-AG: $t_{21}$ = 0.32, NS) without altering dialysate AEA or OEA levels in this part of the mPFC (AEA: $t_{21}$ = 0.73, NS; OEA: $t_{21}$ = -0.32, NS). Finally, Pearson's correlation analyses indicated no significant correlations between reward seeking behavior and any neurochemical changes, nor between the different neurochemical effects, for either heroin or sucrose rats (data not shown).
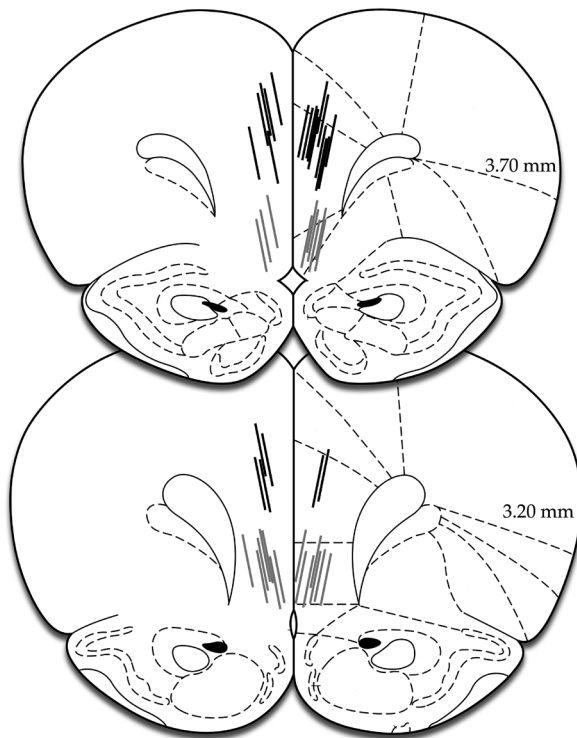


**Figure 5.7. Assessment of microdialysis probe placement.** Schematic drawing of coronal sections of the rat brain depicting probe placements into either the dorsal (black lines) or ventral (grey lines) parts of the mPFC of rats used in the microdialysis studies. Although probe placements within each test group were randomized between both hemispheres, for simplicity reasons, placements for sucrose and heroin rats are here depicted in the left and right hemispheres, respectively. Numbers indicate anterior distance from bregma. Pictures are adapted from Paxinos & Watson (1998).

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*Histology and exclusions*
Figure 7 depicts the probe placements for all animals that were included in the microdialysis experiments. In the heroin experiments, 31 rats implanted with a microdialysis probe directed at either the dmPFC ($n$ = 16) or vmPFC ($n$ = 15) were successfully tested for relapse to heroin seeking. Of those rats, in total five rats were excluded from the analyses. One rat was excluded from the dmPFC group as for this animal histological examination showed probe placement outside the borders of the mPFC, whereas an additional four rats (two from each group) were excluded due to abnormal and fluctuating baseline dialysate content. Finally, the AEA data of one rat in the heroin dmPFC was excluded due to interfering peaks in the mass spectra. In the sucrose experiments, 23 rats implanted with a microdialysis probe directed at either the dmPFC ($n$ = 10) or vmPFC ($n$ = 13) were successfully tested for relapse to sucrose seeking. However, three individuals had to be excluded from the vmPFC group: one as for this animal histological examination showed probe placement outside the borders of the mPFC, another one due to the detection of a considerable lesion surrounding the microdialysis probe, and a third rat due to abnormal and fluctuating baseline dialysate analyte values.

## Discussion

The eCB system, and particularly CB1 receptor activity, is generally thought to be involved in cue- and priming-induced, but not stress-induced, reinstatement of drug and sucrose seeking (De Vries and Schoffelmeer, 2005; Fattore et al., 2007; Serrano and Parsons, 2011). Here we demonstrated that the attenuating effects of the CB1 receptor antagonist SR141716A on relapse to heroin and sucrose seeking are replicated by the neutral CB1 receptor antagonist O-2050. In contrast to CB1 receptor blockade, FAAH inhibition (by URB597) did not affect heroin or sucrose seeking. Finally, we provided the first evidence that heroin seeking results in increased interstitial 2-AG levels that are most pronounced in the dorsal medial prefrontal cortex (dmPFC) along with delayed post-seeking changes in interstitial AEA and OEA levels specifically in the dmPFC. These effects were not fully replicated by sucrose seeking, which produced lesser increases in dmPFC 2-AG levels and did not alter AEA or OEA levels in either region evaluated.

*Post-abstinence relapse to reward seeking requires activation of the endocannabinoid system*
The results with the neutral CB1 receptor antagonist O-2050 indicated that relapse-suppressing effects of traditional CB1 receptor antagonists such as SR141716A and AM251 result from blockade of eCB-induced CB1 receptor activation rather than the putative inverse agonist properties of those compounds (Pertwee, 2005). The subsequent observation that the FAAH inhibitor URB597 affected neither heroin nor sucrose seeking suggests that FAAH activity does not play a critical role during relapse behavior. Intriguingly, others have recently shown that URB597 attenuated cue-induced reinstatement of nicotine and cocaine seeking, but not alcohol seeking, in rats (Cippitelli

et al., 2008; Adamczyk et al., 2009; Forget et al., 2009). However, these URB597-effects may at least partly be independent of CB1 receptor activation and mediated by non-CB1 receptor-targeting FAAH-substrates including OEA (Melis et al., 2008; Mascia et al., 2011). The lack of effect of URB597 on conditioned heroin and sucrose seeking observed here combined with the absence of robust changes in mPFC OEA release during the relapse sessions extend previous findings and suggest that this particular mechanism may be restricted to psychostimulant seeking.

A lack of acute effects of URB597 on relapse behavior fits with the conclusion that 2-AG is the primary eCB mediating relapse behavior, as suggested by the microdialysis results. In particular, relapse to heroin seeking was associated with a robust increase in interstitial 2-AG levels during the elicitation of behavior with no concurrent alteration in interstitial AEA levels. The heroin seeking-associated enhancement of 2-AG release was most pronounced in the dmPFC, where this effect was significantly greater than that observed during sucrose seeking. Because augmented 2-AG release will result in increased CB1 receptor activation, our data are in line with previous reports that conditioned drug seeking is attenuated by intra-mPFC CB1 receptor antagonist administration (Kodas et al., 2007; Alvarez-Jaimes et al., 2008; Hiranita et al., 2008). In contrast to 2-AG, dialysate AEA and OEA levels were not altered during relapse testing in any of the four experimental groups. However, a significant decline in dialysate AEA and OEA content, suggestive of increased FAAH activity, was evident selectively in dmPFC samples collected after termination of heroin seeking. This post-session decrease in AEA and OEA levels might have resulted from relapse-associated stress (Goeders and Clampitt, 2002) in light of recent evidence of stress-induced decreases in mPFC AEA and OEA content (McLaughlin et al., 2012). The post-session decline in AEA might have also contributed to the post-session elevations in dmPFC 2-AG levels (e.g. Maccarrone et al., 2008) that appeared to be more prolonged following heroin seeking.

The functional significance of the observed alterations in mPFC eCB release to the display of relapse behavior as well as the underlying mechanism remains to be determined. To this end, experiments involving intra-mPFC manipulations of 2-AG levels would be an important first step. Unfortunately, such experiments are currently still hampered by a lack of selective and potent 2-AG synthesis and hydrolysis inhibitors suitable for *in vivo* use in rats (Wiskerke et al., 2012). Although rather circumstantial, the neurochemical changes did not appear to directly correlate with the displayed drug seeking behavior. Moreover, distinct patterns of *in vivo* eCB release were found in heroin- and sucrose-trained rats, while all groups of rats displayed similar relapse behavior. Hence, it is conceivable that drug seeking-associated eCB release is not related to lever pressing, i.e. motor activity, per se. This conclusion was further substantiated by the observation that particularly the changes in eCB release in the dmPFC of heroin-trained rats exacerbated following completion of the relapse test, when rats had been returned to their homecages. In our experiments the relapse tests basically constituted a first cue+context extinction session, i.e. the first session during which the rats learned that the reward-associated cues and context no longer predicted actual reward delivery upon active lever pressing. In view of the ongoing debate on the

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involvement of the (mPFC) eCB system in extinction learning (Marsicano et al., 2002; Lin et al., 2009; Hernandez and Cheer, 2011), the observed changes in eCB release may be related to the onset of extinction learning. However, given the relapse suppressing-effects of CB1 receptor antagonists, perhaps a more likely explanation may be that eCBs mediate retrieval of conditioned drug-related memories or other cognitive processes leading up to relapse.

*Endogenous cannabinoids, the medial prefrontal cortex, and relapse to drug seeking*
Although we cannot rule out involvement of other brain regions such as the nucleus accumbens and basolateral amygdala (Kodas et al., 2007; Alvarez-Jaimes et al., 2008; Hiranita et al., 2008), our microdialysis data point toward the mPFC, and particularly the dmPFC, as a primary anatomical locus where eCBs modulate relapse behavior. Previous microdialysis and electrophysiological studies have suggested that the *net* effect of acute CB1 receptor stimulation in the mPFC is a decrease in GABAergic signaling, and consequently, enhanced activity of glutamatergic pyramidal output neurons (Pistis et al., 2001, 2002; Egerton et al., 2006). Although additional receptors, including acetylcholinergic nicotinic receptors (Hiranita et al., 2008), may also be involved, the above-described mechanism provides a likely explanation for how dmPFC 2-AG may drive drug seeking. Indeed, human imaging studies have found enhanced activity in the PFC during cue-elicited drug craving, including heroin craving (Grant et al., 1996; Langleben et al., 2008; Goldstein and Volkow, 2011). Similarly, it is well known that increased activity of pyramidal neurons in the dmPFC, resulting in increased glutamate release into downstream brain regions (mainly the nucleus accumbens core), drives cue- and drug-induced drug seeking in rats (McFarland and Kalivas, 2001; McLaughlin and See, 2003; LaLumiere and Kalivas, 2008). In support of our hypothesis, systemic CB1 receptor blockade has previously been found to attenuate cocaine-induced glutamate release in the rat nucleus accumbens core (Xi et al., 2006).

*The endocannabinoid system: a pharmacological target for the treatment of relapse to reward seeking in general?*
Although reinstatement of extinguished drug seeking has been shown to depend on CB1 receptor activity, our study is the first to demonstrate that the effectiveness of CB1 antagonists does not depend on prior extinction training. Moreover, we showed that the mPFC eCB system is activated upon post-abstinence relapse to drug seeking. These notions are important because there is evidence that the role of the mPFC in relapse behavior may change following extinction training (Peters et al., 2008; Koya et al., 2009; but see Rogers et al., 2008; Van den Oever et al., 2008). It should be stressed that because rats in our study were re-exposed to both the drug-associated context and discrete cues during the relapse test, the current results do not allow for any conclusions regarding the precise stimuli that trigger intra-mPFC eCB release. In this regard, previous studies using systemic administration of CB1 receptor antagonists have shown efficacy of such compounds in suppressing drug seeking behavior induced by either contextual or discrete cues (De Vries et al., 2001; Diergaarde et al., 2008a). It also remains to be determined whether eCB-

related mechanisms underlying cue-elicited relapse behavior generalize across different reinforcers as would be suggested by results from previous studies employing systemic or intracranial administration of CB1 receptor antagonists (De Vries and Schoffelmeer, 2005; Fattore et al., 2007; Serrano and Parsons, 2011). Intriguingly, we observed that conditioned heroin and sucrose seeking were associated with distinct patterns of mPFC eCB release, whereas both behaviors can effectively be suppressed by systemic administration of a CB1 receptor antagonist (current study; De Vries et al., 2003, 2005). Thus, although at the systems level CB1 receptor activation likely plays a role in general reward seeking, the underlying eCB-mediated mechanisms may be reinforcer-specific.

*Concluding remarks*
In summary, the current study demonstrated that relapse to heroin and sucrose seeking critically depends on eCB-mediated CB1 receptor activation. Moreover, where previous studies had already pinpointed the mPFC as an important brain region where eCBs may regulate relapse behavior, we provided evidence for relapse-related 2-AG release in this brain area. Since traditional CB1 receptor antagonists are no longer thought to be viable pharmacotherapeutics due to adverse side-effects, gaining knowledge on how eCBs mediate relapse may provide opportunities for second generation cannabinoid-based anti-addiction pharmacotherapies.

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