Specific and converging modulation of the CRH and noradrenergic systems across drug classes and natural rewards after short- and long-term withdrawal

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ABSTRACT

Stress is known to play a critical role in relapse to drug use as well as in food craving. Craving itself is a key determinant of relapse, and cue-induced drug craving has been shown to increase, or ‘incubate’, over time for certain drugs such as cocaine and nicotine, though this effect is less consistent for others such as opiates. However, the modulations of stress-related biochemical systems after early or protracted withdrawal that could contribute to this incubation phenomenon have not yet been systematically examined in animal models, nor has the specificity of these mechanisms been tested across different drug classes or reinforcers. To address this gap, we analysed brains from male Wistar rats that self-administered cocaine (0.75 mg/kg, i.v.), heroin (0.075 mg/kg, i.v.), or saline, and subsequently assessed changes in plasma corticosterone, ornithine and other stress-related amines, alongside central gene and protein expression (CRH, CRH2 receptor, and α- and β-adrenergic receptor subunits) after 1 or 30 days of withdrawal. A parallel experiment was conducted using sucrose as a reinforcer. Our findings indicate that although most effects were reinforcer-specific, convergent adaptations were also observed, particularly within noradrenergic systems and the basolateral amygdala, suggesting that these mechanisms may represent a potential target for pharmacological interventions to prevent relapse.

1. INTRODUCTION

Addiction, or substance use disorder according to the DSM-5-TR terminology, has been frequently defined as a chronic relapsing disorder (Blithikioti *et al.* 2025; Guerrin *et al.* 2025; Leukefeld and Tims 1989). As such, it is a crucial research goal to obtain a deep and comprehensive understanding of the mechanisms involved in relapse. Among other factors, stress plays a crucial role in relapse into drug use (LaFond *et al.* 2024; Sinha 2024; Carmody 1990). There is ample preclinical literature which indicates that rodents will reinstate their drug seeking behaviour after exposure to different stressors (Mantsch *et al.* 2016). Moreover, a previous stressful experience can facilitate reinstatement of drug-seeking behaviour (Morales-Silva *et al.* 2024). In humans, early clinical reports showed that stress facilitates relapse (Carmody 1990; Wallace 1989), and numerous preclinical studies have examined the neural and endocrine bases of the phenomenon (Mantsch *et al.* 2016). An important factor driving relapse is craving (Vafaie and Kober 2022) which is a time-dependent phenomenon. Indeed, while basal craving diminishes over time, cue-induced craving increases ('incubates') over withdrawal (wd) time in cocaine users (Gawin and Kleber 1986). Importantly, this phenomenon seems to be absent in opioid users (Bergeria *et al.* 2024) which underscores the importance of comparing across drug classes in incubation studies. While a tremendous effort has been made to search for time-dependent neurobiological mechanisms of this incubation phenomenon (Chow *et al.* 2025) with potential targets for treatment being identified (Liu *et al.* 2023), the role of stress in this paradigm and their associated biochemical mechanisms, such as the noradrenaline (NA) system and hypothalamic-pituitary-adrenal (HPA) axis, are still poorly understood (Venniro *et al.* 2021).

In addition to comparing a psychostimulant drug (cocaine) with an opioid drug (heroin) in this work we also introduced a third rewarding substance, sucrose-sweetened water. An incubation phenomenon similar to the one observed for cocaine has also been described for sucrose-sweetened water (Grimm 2020; Grimm *et al.* 2002) and human studies have also reported incubation of food craving (Ruiz *et al.* 2024; Coutinho *et al.* 2018) which underscores the importance of including this third reward as a comparator and also due to its own significance. We have focused on the corticotrophin releasing hormone (CRH) system and NA systems as the main mechanisms of the stress response and due to their implications in craving in humans (Varodayan *et al.* 2025) and the lack of data on the role of these systems in the incubation of seeking across drug classes and different rewards. In addition to measuring CRH- and NA-related parameters in cortico-

striato-limbic regions involved in the incubation phenomenon (Chow *et al.* 2025) and study their potential correlations as we have done in previous works (Roura-Martínez *et al.* 2020), here we have also measured peripheral mediators such as corticosterone and different amino acids relevant to metabolic processes involved in the stress response.

We show that incubation of cocaine, heroin, and sucrose seeking involves early, late, and persistent stress-related alterations, largely substance-specific but converging on *Adra2a* gene in the nucleus accumbens core and *Adrb1* in the basolateral amygdala (BLA). This is the first report using the incubation of seeking paradigm focusing on the CRH and NA systems, addressing a significant gap in the literature.

2. EXPERIMENTAL PROCEDURES

Animals. We used 84 Lewis male rats (300-320 g at the beginning of the experiments) purchased from Harlan International Ibérica. The self-administration behaviour of these animals has been described in a previous publication (see Roura-Martínez et al., (2020b) for more details) displaying patterns of drug or sucrose intake previously shown to produce incubation of seeking.

Experimental design. One batch of rats was submitted to jugular catheter surgery (for drug or saline self-administration) and the other left intact (sucrose/water self-administration). After ten days of drug/saline or sucrose/water self-administration, the rats underwent 1 or 30 days of forced wd with regular handling. The rats that received intravenous administration of cocaine, heroin, or saline (iv control) were segregated into six groups (3 substances × 2 wd periods; n=8 rats per group), while the rats that received sucrose or water (oral control) were distributed in four groups (2 substances × 2 wd periods; n=9 rats per group).

Surgical procedure. An intravenous polyvinylchloride tubing (0.064 mm i.d.) catheter was implanted into the right jugular vein at approximately the level of the atrium and passed subcutaneously to exit the midscapular region (Higuera-Matas *et al.* 2008). Surgical procedures were performed under isoflurane gas anaesthesia (5% for induction and 2% for maintenance) and buprenorphine analgesia, and the catheters were flushed daily with 0.5 mL of gentamicin (40 mg/mL) dissolved in heparinized saline in order to prevent infection and to maintain patency (see (Roura-Martínez *et al.* 2020)).

Self-administration. Self-administration sessions were performed in Skinner boxes (Coulbourn Instruments or Med-Associates) and two levers (active and inactive) monitored with Med-PC software. The house light was off during the sessions, and the door of the sound-attenuating cubicle left ajar, allowing some environmental light from the room. Each time the active lever was pressed (fixed-ratio 1), a pump outside the box was switched on for 5 seconds and either the drug or saline solution was infused through the catheter, or the sucrose solution or water was dispensed into a receptacle placed in between the levers. A cue-light over the active lever switched on for 10 seconds at the same time. Subsequently, there was a time-out period of 40 seconds with no programmed consequences. Cocaine, heroin, or saline self-administration sessions lasted 6 hours per day, as described previously.(Roura-Martínez *et al.* 2020). Rats orally self-administering sucrose or water were subjected to shorter sessions (2 h/d). The doses per injection used in the experiments were 0.075 mg/kg of heroin; 0.75 mg/kg of cocaine-HCl; and 10% w/v sucrose (Sigma-Aldrich S1888), diluted in 0.1 mL of saline (0.9%, NaCl physiological saline. To facilitate the acquisition of this behaviour, we placed two sucrose pellets on the active lever in the first two self-administration sessions.

Animal sacrifice and tissue processing. We weighed and sacrificed the rats after 1 or 30 days of wd by decapitation, between 11:00 and 13:00. Immediately after, the brain was removed in about one minute, frozen by ten seconds of immersion in isopentane (VWR 24872.298) cooled with dry ice, and stored at -70°C. We collected trunk blood in a heparinized tube and extracted plasma by centrifugation at 1000g for 10 min at 4 °C, then stored at -70°C. Adrenal glands were removed and weighed.

Brain dissection. Brain was tempered 1 h at -20°C in the cryostat chamber (Microm, Cryostat HM 500O) embedded in TissueTek (Sakura, 4583). We collected 300µm thickness slices on a sterile cold surface and dissected them with a lancet, according to a rat neuroanatomical atlas (Paxinos and Watson, 2007; regions depicted in Figure 6). We stored the tissue during dissection in sterile tubes of 0.2 mL in dry ice and then at -70°C.

Tissue homogenization. We weighed and homogenized the samples in HEPES buffer (50 mM, pH 7.5) prepared in DEPC-treated water, containing sucrose (320 mM), protease and phosphatase inhibitors, and sodium butyrate (20 mM) using a motorized mortar (Sigma-Aldrich, pellet pestle Z359971). After being kept on ice for 10 min they were centrifuged at 1000 g for 10 min at 4°C. A volume of supernatant equivalent to 3-4 mg of

original tissue (≤80 µL) was added to 800 µL of QIAzol (Qiagen 79306) for extraction of cytoplasmic RNA. Another volume was added to loading buffer (Tris 62.5 mM pH 6.8, glycerol 20% v/v, SDS 2% w/v, dithiothreitol 50 mM) for Western blot. Then, we stored the samples at -70°C.

Level of plasma corticosterone by radioimmunoassay. The plasmas were thawed, centrifuged at 10,000 g for 10 min at 4°C to discard precipitates, tempered at room temperature (RT), and then analysed using the Coat-a-Count® Rat Corticosterone kit (Siemens). Briefly, 50µL of sample or calibrator was added to tubes covered with anti-corticosterone antibody. Then, 1 mL of I^{125}-corticosterone solution was added, vortexed and incubated at RT for 2 h. The content was then decanted and the signal analysed (1 min/tube, in counts per minute, cpm) in a gamma counter (Wallac, Automatic Gamma Counter 1470 Wizard). To estimate the non-specific binding, samples or calibrators in non-covered tubes were measured. The maximum binding value was estimated by measuring a calibrated tube without labelled corticosterone. The corticosterone values were interpolated in a semi-logarithmic line plotted against the logarithm of the concentration with the binding value and expressed in ng/mL.

Level of plasma amines by capillary electrophoresis. The content of amines was analysed according to the method described in Lorenzo et al. (2013) (Lorenzo *et al*. 2013). Samples were passed through a 0.22 µm filter (VWR 28145-491 13 mm syringe filter 0.2 µm PTFE membrane) and then stored at -70°C until use. In parallel, cocktails of different amines were prepared to be used as a calibration curve (from Sigma: L-glutamic acid (49449), glycine (G7126), L-glutamine (49419), taurine (T0625), L-serine (S4500), D-serine (S4250), L-proline (81709), L-isoleucine (W527602), L-ornithine (O2375), L-threonine (T8625)). L-2-aminoadipic acid (Sigma, A7275) was used as internal standard (IS). In each cocktail, the concentration of each amine was different, but the total concentration of amines was maintained constant. The derivatization (20 µL sample, 20 µL 200 mM IS, 25 µL 40 mM 4-fluoro-7-nitro-benzofurazan (Alfa-Aesar J61336), 150 µL 10 mM borate buffer pH 10.0) was carried out at 60°C for 15 min in darkness. Then, the samples were stored in the chamber of the capillary electrophoresis apparatus (Beckman Coulter PA 800 plus) at 7°C for at least 30 min before the start of the electrophoresis, carried out at 17°C (electrophoresis buffer: 175 mM borate buffer pH 10.25, 12.5 mM β-cyclodextrin (SAFC W40,282-6)). Before its first use, the capillary column of silica (length: 60cm; inner diameter: 75µm) was conditioned with 1 M NaOH (15 min) and water (15 min),

and between electrophoresis with 0.1 M HCl (3 min), water (5 min) and electrophoresis buffer (5 min). The sample was injected at the anode at 0.5 psi (33 mbar) for 10 seconds. A potential difference of 21 kV was applied, observing currents of 120 µA. The molecules were detected at the cathode by means of laser induced fluorescence (LIF), exciting at 488 nm (argon lamp) and detecting the emission at 522 nm. To obtain reproducible electropherograms, the electrophoresis buffer was renewed every six analyses. In each session, a calibration curve was also injected. The electropherograms were analysed with the 32 KaratTM software, using the corrected area normalized to the internal standard. The values were expressed as pmol/mL.

Isolation of RNA. The RNA was extracted using a protocol adapted from Chomczynski and Sacchi (Chomczynski and Sacchi 1987; Chomczynski and Sacchi 2006). Briefly, the sample (stored in QIAzol) was thawed and left at RT for at least 5 min. 160 µL of chloroform (Merck 1.02445.2500) were added, mixed for 15 seconds and incubated at RT for 2-3 min. It was then centrifuged at 12,000 g for 15 min at 4°C. The upper (aqueous) phase was transferred to a new sterile 1.5 mL tube, with 400 µL of isopropanol (Fischer Scientific, BP2618) and 10 µg of glycogen (Sigma G1767), mixed and incubated at RT for 10 min. It was centrifuged at 12000 g for 10 min at 4°C and the supernatant discarded. The precipitate was washed twice in 1 mL of cold (-20°C) 75% v/v ethanol and centrifuged at 7500g for 5 min at 4°C, discarding the supernatants. Finally, the precipitate was allowed to air-dry, resuspended in nuclease-free water and stored at -70°C. The RNA was quantified in a Bioanalizer 2100 system (Agilent, RNA Nano Chips, 5067-1511), with values of RIN≥7.0. Unless exceptions, absorbance ratios 260nm/280nm ≥1.8 were obtained. An isolation efficiency of 0.7-1.0 µg RNA/mg tissue was obtained.

RT-qPCR. About 250-500ng RNA (depending on the region) was treated with DNaseI (Invitrogen, 18068-015), retrotranscribed (Biorad, iScript cDNA Synthesis kit, 170-8891), diluted 1:10 in nuclease-free water and stored at -70°C. Gene expression was analysed using SYBR Green (Biorad, SsoAdvanced Universal SYBR Green Supermix), in a CFX96 C1000 Touch of Biorad system. The total reaction volume was 10µL, and the primers were in the range of 500-750nM. The thermal protocol was as follows: 5 min at 95°C, 40 cycles of 5 min at 95°C, and 30 seconds at 60°C, followed by a melting curve. We calculated the Ct values and the efficiencies with the free software LinRegPCR (Ruijter *et al.* 2009) and then calculated the fold change for each sample and gene according to Pfaffl (Pfaffl 2001), using *Gapdh* as housekeeping gene (which did not vary across conditions). Because

adrenoceptors have different (and sometimes opposite) effects in different brain regions, we also calculated ratios between receptors for each region and ratios between regions for each receptor.

Primer list

α1 adrenoceptor (*Adra1)*.

S: 5'-ctcgagagaagaaagctgcca-3' A: 5'-aaaacggtttccgaaggcttg-3'

α2A Adrenoceptor (*Adra2a*).

S: 5'-tcctgagagggaagggattt-3' A: 5'-agttactggggcaagtggtg-3'

β1 adrenoceptor (*Adrb1*).

S: 5'-gctctggacttcggtagacg-3' A: '-acttggggtcgttgtagcag-3'

Protein quantification. Protein content was quantified in microplates using the colorimetric method described by Bradford (Bradford 1976), with minor modifications. Briefly, 250 µL of the reagent (a 1:5 dilution of the Bio-Rad Protein Assay Dye Reagent Concentrate 500-0006) was added to 5 µL of the sample. A calibration curve of BSA was made. The 595nm/450nm absorbance ratio (quantified in an Asys Hitech DigiScan) was used to interpolate unknown values.

Western blot. 5 µg of protein from each sample was resolved on 12% SDS-PAGE gels in a MiniProtean® Tetra System (Bio-Rad). To quantify the total protein, the gels were exposed to UV light for about 1 min to activate the reaction between trichloroethanol and tryptophan (Ladner *et al.* 2004), and they were then recorded on an Amersham Imager 600 (General Electrics). The proteins were transferred to 0.2 µm PVDF membranes (Trans-Blot® TurboTM transfer pack, Bio-Rad) in a Trans-Blot® Turbo transfer system (Biorad), blocked with 5% w/v BSA in TBST for 1 h at RT and then probed overnight in TBST at 4 ºC with the primary anti-CRH antibody (Abcam, ab184238) or anti- CRH receptor 2 (Abcam, ab104368) diluted 1:3.000. Antibody binding was recognized for one hour at RT with an HRP-conjugated anti-rabbit IgG secondary antibody (Abcam ab6721) diluted 1:5,000, and the signal was developed using ECL2 and recorded on an Amersham Imager 600. Proteins were analysed by densitometry using ImageJ free software and the data were normalized to total protein and by gel according to the criterion described by Degasperi (Degasperi *et al.* 2014).

Missing samples. Due to storage problems, two brains were lost (water wd1 group).

Univariate analysis. We carried out two-way ANOVA (IBM Statistics) to compare specific biochemical parameters (log or inverse transformation when necessary) between groups due to the drug exposure -drug- (cocaine, heroin or saline), sucrose exposure -sucrose- (sucrose vs water) and/or wd (wd1 vs wd30). Interactions were analysed by simple effects analysis (Sydak correction). Following a significant effect of the treatment, we performed the Ryan post hoc procedure (REGWQ) to allocate the differences between groups (Field 2013).

Bivariate statistics. To detect correlated variables that could have a psychobiological meaning, a screening of Pearson correlations (IBM Statistics) followed by their respective comparisons between groups was performed. The Fisher transformation was applied to the r-value, and then the Z-value and its p-value were calculated to compare the correlations between groups. We made two types of comparisons for each substance: a) the wd1 group versus its wd1 control and its wd30 group, and b) the wd30 group versus its wd30 control and its wd1 group. To select the correlations with differences between groups, no multiple comparisons correction was applied but we filtered them on the basis of three criteria: 1) $n>5$ in the three compared groups; 2) a significant r ($p<0.05$) in the group to be tested; and 3) a significant Z ($p<0.05$) for comparisons.

3. RESULTS

For all variables, descriptive statistics (N, mean, and SD per group) and inferential results are presented in Tables 1 (saline, cocaine, heroin) and 2 (water, sucrose). Table 3 shows the parameters from the correlation analyses. For the sake of conciseness, we only report here the statistics of the most relevant interactions and post-hoc tests.

The behavioural data from these animals has been published elsewhere (Roura-Martínez *et al.* 2020). Importantly, rats had self-administration patterns that were undistinguishable from those of rats displaying the incubation phenomenon (i.e. more lever presses under extinction conditions in wd30 compared to wd1), for all the reinforcers tested (cocaine, heroin and sucrose) (see Roura-Martínez et al. (2020) for further details).

A. Stress-related peripheral parameters

Body weight.

Differences in body weight were only attributable to the passage of time (main effect of wd), with no effects of drug or sucrose nor of their interaction with wd time (Tables 1 and 2).

Adrenal gland index.

In parallel with weight gain, the adrenal gland index decreased over time. In addition, this index was higher in the cocaine and heroin groups during early wd (wd1) (drug × wd: $F(2,42)=5.68$, $p=0.007$, $\eta^2p=0.21$; saline wd1 vs cocaine wd1, $p<0.001$; saline wd1 vs heroin wd1, $p<0.001$). We did not detect any significant differences in the sucrose experiment (Fig. 1A and B; See Tables 1 and 2 for further details).

Corticosterone.

We observed a significant drug × wd interaction ($F(2,42)=3.32$, $p=0.046$, $\eta^2p=0.14$) which, upon further analysis, showed that plasma corticosterone levels were significantly higher in the heroin group compared with the saline group during early wd (saline wd1 vs heroin wd1, $p=0.033$). Moreover, a significant effect of wd was observed in the heroin group (Fig. 1C), with a reduction in corticosterone levels over time (heroin wd1 vs heroin wd30, $p=0.004$). There were no effects in the sucrose/water experiment (Fig. 1D) (Tables 1 and 2).

Plasma amino acid levels and L-ornithine metabolism.

Among the amines analysed, we found significant modulations in those related to L-ornithine metabolism. This amino acid is metabolically linked to L-glutamate, L-glutamine, and L-proline (Watford 2008), and is also associated with stress (see Discussion). We analysed both the concentrations of these amines individually and the ratios of L-ornithine to each of the others. Cocaine, heroin, or sucrose self-administration did not alter absolute concentrations (Tables 1 and 2). However, when examining ratios, we found an increment in the L-ornithine/L-glutamine ratio for heroin at early wd compared with saline and decreases across wd for both drugs (significant drug × wd interaction: $F(2,41)=3.77$, $p=0.031$, $\eta^2p=0.16$; saline wd1 vs heroin wd1, $p=0.024$; cocaine wd1 vs cocaine wd30, $p=0.008$; heroin wd1 vs heroin wd30, $p<.001$) (Fig. 1E). Similarly, the L-ornithine/L-proline ratio increased at early wd but decreased after one month of wd in heroin rats (drug × wd: $F(2,41)=3.71$, $p=0.033$, $\eta^2p=0.15$; saline wd1 vs heroin wd1, $p=0.048$; heroin wd1 vs her-

oin wd30, p=0.032 Fig. 1G). No significant effects were observed in the sucrose/water experiment (Fig. 1F and 1H; Tables 1 and 2).

B. Stress-related central parameters

Enduring self-administration effects

All three rewarding substances tested (cocaine, heroin and sucrose) produced long-lasting effects in the nucleus accumbens. In terms of gene expression, cocaine self-administration was associated with a trend towards decreased *Adra2a* expression levels in the shell (Fig. 2C; drug effect: $F(2,38)=3.55$, $p=0.039$, $\eta^2p=.16$; saline vs cocaine, $p=0.068$) and an increase in the *Adra2a* core/shell ratio (Fig. 2G; drug effect: $F(2,36)=3.65$, $p=0.036$, $\eta^2p=.17$; saline vs cocaine, $p=0.033$). This ratio also increased after sucrose consumption (Fig. 2H; sucrose effect: $F(1,19)=8.18$, $p=0.010$, $\eta^2p=0.30$), accompanied by additional changes in the core: decreases in *Adra1* (Fig. 2B; sucrose effect: $F(1,30)=9.28$, $p=0.005$, $\eta^2p=0.24$) and increases in the *Adra2a/Adra1* ratio (Fig. 2F; sucrose effect: $F(1,21)=7.82$, $p=0.011$, $\eta^2p=0.27$).

In the prefrontal cortex, sucrose self-administration also produced lasting changes in the dmPFC: decreases in *Adrb1* (Fig. 3F; sucrose effect: $F(1,30)=21.40$, $p<0.001$, $\eta^2p=0.42$), *Adra1* (Fig. 3B; sucrose effect: $F(1,30)=9.96$, $p=0.004$, $\eta^2p=0.25$), *Adra2a* (Fig. 3D; sucrose effect: $F(1,30)=8.48$, $p=0.007$, $\eta^2p=0.22$), *Adra1/Adrb1* ratio (Fig. 4F; sucrose effect: $F(1,30)=24.37$, $p<.001$, $\eta^2p=0.45$), and *Adra1* dmPFC/vmPFC ratio (Fig. 4H; sucrose effect: $F(1,30)=10.14$, $p=0.003$, $\eta^2p=.26$); and increases in the *Adra2a/Adra1* (Fig. 4B; sucrose effect: $F(1,30)=29.38$, $p<.001$, $\eta^2p=.50$), *Adra2a* BLA/dmPFC ratio (Fig. 4J; sucrose effect: $F(1,22)=8.94$, $p=0.007$, $\eta^2p=0.29$), and dmPFC *Adra2a*/*Adra1* ratio (Fig. 4D; sucrose effect: $F(1,30)=4.53$, $p=0.042$, $\eta^2p=0.13$).

We then computed correlations between measured parameters and examined their modulation by drug/sucrose self-administration and wd (Table 3). In the nucleus accumbens core, CRH and *Adra2a* levels were negatively correlated in saline groups (saline wd1, $r=–0.949$, $p=0.001$; saline wd30, $r=–0.779$, $p=0.023$). This correlation was abolished following cocaine (as evidenced by the significant difference among the correlations revealed by the differences among z scores) (wd1: $Z=–2.533$, $p=0.011$; wd30: $Z=–3.079$, $p=0.002$), and also by heroin self-administration (wd1: $Z=–2.411$, $p=0.016$; wd30: $Z=–1.983$, $p=0.047$) (Fig. 5).

Modulations occurring across withdrawal

Specific dmPFC alterations that were emerging after sucrose self-administration, albeit non-significantly, became significant after one month of wd. These changes included decreases in *Adrb1* gene expression (Fig. 3F; sucrose × wd: $F(1,30)=5.93$, $p=0.021$, $\eta^2p=0.17$; water wd30 vs sucrose wd30, $p<0.001$; sucrose wd1 vs sucrose wd30, $p<0.001$), a trend for a decreases in the *Adra1* dmPFC/vmPFC ratio (Fig. 4H; sucrose × wd: $F(1,29)=3.90$, $p=0.058$, $\eta^2p=0.12$; water wd30 vs sucrose wd30, $p=0.001$; sucrose wd1 vs sucrose wd30, $p=0.002$), and reductions in the *Adra1*/*Adrb1* ratio (Fig. 4F; sucrose × wd: $F(1,30)=4.68$, $p=0.039$, $\eta^2p=0.14$; trend in water wd1 vs sucrose wd1, $p=0.051$; water wd30 vs sucrose wd30, $p<0.001$; sucrose wd1 vs sucrose wd30, $p<0.001$); In addition, there were also increments in the *Adra2a*/*Adra1* ratio (Fig. 4B; sucrose × wd: $F(1,30)=11.69$, $p=0.002$, $\eta^2p=0.28$; water wd30 vs sucrose wd30, $p<0.001$; sucrose wd1 vs sucrose wd30, $p<0.001$). Moreover, the *Adra1*/*Adrb1* ratio also increased after one month of heroin wd (Fig. 4E; drug × wd $F(2,42)=3.34$, $p=0.045$, $\eta^2p=0.14$; saline wd30 vs heroin wd30, $p=0.016$).

When we analysed the patterns of correlations across wd, we observed, in the nucleus accumbens core, a significant correlation between CRH and *Adra2a*, specifically in the cocaine wd30 group ($r=0.77,1$ $p=0.042$). Most importantly, we found a common node of changes in correlations across all reinforcers, centred on *Adrb1* expression in the BLA. In addition, we found that sucrose self-administration and wd modified the correlations between *Adrb1* in BLA and CRHR2 in the vmPFC, as evidenced by the significant difference between z scores (water wd1 $=0.107$, $p=0.819$; sucrose wd1, $r=0.907$, $p=0.005$; wd1, water vs sucrose, $Z=–1.983$, $p=0.047$; sucrose wd1 vs sucrose wd30, $Z=2.697$, $p=0.007$), and that cocaine also modified the correlations between BLA *Adrb1* expression levels and CRHR2 accumulation in the dmPFC (saline wd30 $r=0.001$, $p=0.998$; cocaine wd30, $r=0.859$, $p=0.006$; wd30, saline vs cocaine, $Z=–2.038$, $p=0.042$; cocaine wd1 vs wd30, $Z=–2.662$, $p=0.008$). In addition, the correlation between BLA *Adrb1* and CRHR2 levels changed during cocaine wd (cocaine wd30, $r=0.944$, $p<0.001$; wd30, saline vs cocaine, $Z=–2.270$, $p=0.023$; cocaine wd1 vs wd30, $Z=–3.060$, $p=0.002$), as was the case also with heroin, which modified correlations in the BLA (heroin wd30, $r=–0.905$, $p=0.005$; wd30, saline vs heroin, $Z=2.471$, $p=0.013$ and a trend in heroin wd1 vs wd30, $Z=1.910$, $p=0.056$). BLA *Adrb1* correlations with *Adrb1* in the dmPFC and with *Adra2a* in the vmPFC also shifted during wd from both drugs of abuse (Fig. 5; Detailed statistics are provided in Table 3).

4. **DISCUSSION**

The main aim of this study was to characterise the modulations in HPA and NA systems occurring during wd from cocaine, heroin and the natural reinforcer sucrose, which may be relevant to the incubation of seeking phenomenon. Using a rat model of self-administration which generated intake patterns previously found to produce incubation of seeking, we identified changes emerging both at early and later stages of wd, as well as persistent effects. Some of these alterations were shared between two substances, but most were substance-specific. For example, during early drug wd (cocaine and heroin), we observed adrenal hypertrophy and disruptions in ornithine metabolism. Nevertheless, common nodes emerged across all reinforcers: *Adra2a* expression in the nucleus accumbens core (long-lasting effects) and *Adrb1* expression in the BLA (late effects) (Fig. 6). This study addresses a gap in the literature, as few investigations on incubation of seeking have examined peripheral or central stress-related parameters, despite the well-established role of stress in modulating drug seeking.

As early as 1986, Gawin and Kleber hypothesised that induced craving could exhibit an incubation phenomenon (as cited in (Li et al., 2016)). However, it was not until the 2010s—approximately a decade after its discovery in animal models—that systematic studies of incubation of craving in humans began. Several works have examined the evolution of cue-induced craving throughout abstinence in individuals addicted to tobacco (Bedi *et al.* 2011), methamphetamine (Wang *et al.* 2013), heroin (Wang *et al.* 2013), alcohol (Wang *et al.* 2012), and cocaine (Parvaz *et al.* 2016b). Overall, basal craving and/or wd symptoms decrease as abstinence progresses, whereas cue-induced craving increases during abstinence before subsiding after several months (incubation of craving). Heroin represents an exception, although the only study began assessments after one month of abstinence rather than within the first week. In another investigation, Nava (2006) reported incubation of craving without differentiating between basal and cue-induced forms (Nava *et al.* 2006). Incubation of basal anxiety and/or depressive symptoms has only been observed in methamphetamine and tobacco users, and no studies have investigated cue-induced anxiety.

With regard to non-drug reinforcers, at least three studies reported that basal craving diminishes during abstinence from restrictive diets (Batra *et al.* 2013; Martin *et al.* 2006; Massey and Hill 2012), whereas cue-induced food craving tends to be higher among

individuals undergoing more restrictive regimes (Boswell and Kober 2016). A related phenomenon, incubation of cue-induced fear responses, has been recognised since the early 20th century (Diven 1937) and is considered necessary for the manifestation of trauma-related symptoms.

Although this gap in human research concerning the relationship between stress and incubation of craving has not yet been bridged using animal models, some studies have addressed the issue. For instance, stress-induced seeking incubates when footshocks are applied following heroin consumption, but not following sucrose intake (Shalev *et al.* 2001), and fails to incubate when yohimbine (an ADRA2 antagonist) is used after methamphetamine administration(Shepard *et al.* 2004). Resistance to punishment has been shown to incubate after histamine administration in cocaine-exposed animals (Gancarz-Kausch *et al.* 2014) and after lithium chloride in sucrose-consuming animals (Harkness *et al.* 2010). Regarding cue-induced incubation of seeking, a negative state appears to enhance seeking and a positive state to reduce it, but only when the state is temporally proximal to the extinction test—indicating a transient effect. Such findings have been reported with caloric restriction (heroin: (D'Cunha *et al.* 2017)), immobilisation (cocaine: (Glynn *et al.* 2018)), and environmental enrichment (cocaine: (Zlebnik and Carroll 2015; Chauvet *et al.* 2012; Ma *et al.* 2016); sucrose: (Glueck *et al.* 2017; Grimm *et al.* 2016b; Grimm *et al.* 2013; Grimm *et al.* 2008)).

Voluntary abstinence protocols have further demonstrated that footshock-induced abstinence prevents incubation of food seeking but not methamphetamine seeking (Torres *et al.* 2017; Krasnova *et al.* 2014; Krasnova *et al.* 2017), and food-induced abstinence prevents incubation of heroin seeking (Venniro *et al.* 2017) but not methamphetamine seeking (Caprioli *et al.* 2015; Venniro *et al.* 2017). The general limitation of these studies is that treatments are rarely compared across both early and late wd. More recently, Nicolas (2022) (C *et al.* 2022) investigated whether anxiety-like symptoms incubate in a rat model. In the novelty-induced hypophagia test—the only test performed and only in males—no effect was detected. Notably, each group comprised a pool of animals that had or had not undergone extinction, conflating basal and cue-induced effects. Thus, the relationship between stress and cue-induced incubation of seeking remains unresolved.

If behavioural data leave this relationship uncertain, what insights are gained from physiological and biochemical parameters? In drug users, consumption-related cues activate both the autonomic nervous system (ANS) and the hypothalamic–pituitary–adrenal

(HPA) axis, with stronger responses in heavy users (Fox *et al.* 2005). Both human and animal studies have shown that drug use itself alters ANS and HPA axis activity (Goldfarb and Sinha 2018; Ulrich-Lai and Herman 2009b). For example, heroin consumption suppresses HPA axis activity, whereas cocaine consumption enhances it. Abstinence from both substances induces HPA hyperactivity (Brown *et al.* 2006). Similarly, rats with extended access to cocaine show higher plasma corticosterone levels than those with restricted access (Mantsch, 2014) . In humans, drug users often exhibit a dysfunctional HPA axis, characterised by elevated basal plasma cortisol and exaggerated cortisol responses (Goldfarb and Sinha 2018).

By contrast, contingent consumption of natural reinforcers reduces ANS and HPA activation, unless cues are presented in the absence of the reinforcer, in which case both systems are activated (Ulrich-Lai and Herman 2009a). Notably, none of the aforementioned human studies on incubation reported parallel incubation of cue-induced cardiovascular stress. In our rat studies, we observed early adrenal hypertrophy after cocaine and heroin, but not sucrose, self-administration—an effect typically associated with chronic stress (Ulrich-Lai and Herman 2009a)—which normalised by one month. Plasma corticosterone levels were transiently elevated following heroin self-administration, consistent with earlier findings for cocaine (Thiel *et al.* 2012). Neither we nor Grimm (Grimm *et al.* 2016a) observed such effects after sucrose self-administration (in their case, animals were anaesthetised before blood collection). These findings suggest that, unlike sucrose, drug self-administration under conditions that produce incubation of seeking is capable of activating the HPA axis in the short term.

Additionally, we observed apparent metabolic increases in plasma L-ornithine coinciding with adrenal hypertrophy after drug consumption. This is noteworthy because a homeostatic loop may exist between glucocorticoids and L-ornithine: oral administration of L-ornithine reduces plasma glucocorticoid levels in stressed mice (Kurata *et al.* 2012) and humans (Miyake *et al.* 2014), and corticosterone elevation in mice is negatively regulated via L-ornithine metabolism (Zamora-González *et al.* 2020). Our findings on L-ornithine in the cocaine and heroin groups may therefore represent a compensatory response to HPA axis activation.

Further evidence for HPA activation derives from endocannabinoid (eCB) alterations in the same animals (Roura-Martínez *et al.* 2020). Both chronic stress and corticosterone administration are known to induce changes in central eCB signalling (Bowles *et al.*

2012; Gray *et al.* 2016; Häring *et al.* 2012; McLaughlin and Gobbi 2012; Ramikie and Patel 2012b), typically decreasing anandamide (AEA) and increasing 2-AG. For example, in the BLA, such manipulations produce long-lasting decreases in AEA tone through FAAH upregulation and transient increases in 2-AG (Ramikie and Patel 2012a). In our previous experiments, we showed persistent eCB alterations detectable from the first day of abstinence and lasting at least one month. Both drugs of abuse reduced C*nr1* expression in the dorsomedial prefrontal cortex (dmPFC). Cocaine additionally decreased *Napepld/Faah* expression in the accumbens shell, whereas heroin increased *Faah* in the ventromedial PFC, consistent with reduced AEA signalling. Surprisingly, sucrose consumption also induced changes in the same direction: increased *Dagla/Mgll* in the dmPFC, reduced *Napepld* in the accumbens core, and increased *Faah* and *Cnr1* in the accumbens shell. These findings suggest increased 2-AG and reduced AEA.

Regarding the central noradrenergic system, Hamed and Boguszewski (2018), using a morphine-conditioned place preference model, reported reduced MHPG (the main noradrenaline metabolite) in the nucleus accumbens, but not in the medial prefrontal cortex or amygdala, after prolonged abstinence. In another study, Shepard (Shepard *et al.* 2004) showed that yohimbine, an ADRA2 antagonist, potentiated methamphetamine seeking but did not produce incubation-like effects across wd days 1, 21, and 51. In our experiments, we observed long-lasting noradrenergic alterations in the nucleus accumbens following all three reinforcers, and late effects after cocaine exposure, primarily involving *Adra2a*. Alterations were also detected in other regions, involving both noradrenergic and corticotropin-releasing hormone receptors. Across all reinforcers, these changes converged on *Adrb1* expression in the BLA, linked to distinct receptor networks in this region and the medial prefrontal cortex (Fig. 6). Both the prefrontal cortex and BLA receive noradrenaline from the locus coeruleus via the dorsal bundle, yet receptor dynamics differ: ADRA2A, a high-affinity receptor active under low arousal, exerts excitatory effects in the PFC but inhibitory effects in the BLA; conversely, ADRB1, a low-affinity receptor active during stress, is inhibitory in the PFC but excitatory in the BLA (Arnsten 2009; Mitrano *et al.* 2012; Buffalari and Grace 2007; Ferry and McGaugh 2008). The potential role of ADRB1, rather than ADRA2A, in incubation is intriguing given that the late positive potential—a neurophysiological marker of incubating craving in abstinent cocaine users (Parvaz *et al.* 2016a)—depends on β-receptor-mediated activation of the amygdala (De Rover *et al.* 2012).

CONCLUSION

In summary, our findings demonstrate that incubation of drug seeking is accompanied by peripheral and central stress-related alterations that are largely substance-specific but converge on common noradrenergic nodes. Short-term activation of the HPA axis, adrenal hypertrophy, and changes in L-ornithine metabolism highlight the interplay between stress and craving. Persistent adaptations in the endocannabinoid and noradrenergic systems further implicate these pathways in the incubation process. Overall, these results underscore stress-related mechanisms as critical modulators of drug seeking and potential targets for intervention.

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CONTRIBUTORS

D.R-M performed the experiments and wrote the first draft of the manuscript.
M.U. contributed to the statistical analysis and the first draft of the manuscript.
M. M-F, C. A C. and I B-Y performed part of the molecular experiments.
A.B. performed the capillary electrophoresis analysis.
E. A. contributed to secure funding and oversaw the project
Alejandro Higuera-Matas designed the experiments, secured funding and wrote the final version of the manuscript.

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FOOTNOTES TO FIGURES AND TABLES

Figure 1. Changes in stress-related peripheral parameters during withdrawal. Levels of adrenal glands index (A, B), plasma corticosterone (C, D), and L-ornithine metabolism ratios (E-H) during cocaine and heroin (A, C, E, G) or sucrose (B, D, F, H) wd. Individual values are presented as well as the mean ± SEM. Differences relative to the control group, *p<0.05, **p<0.01, ***p<0.001; differences relative to the same treatment on different wd days, *f* p<0.05, *ff* p<0.01, *fff* p<0.001; general differences between the two wd days, Δ p<0.05, ΔΔ p<0.01, ΔΔΔ p<0.001.

Figure 2. Changes in gene expression of adrenoceptors in the nucleus accumbens during withdrawal. Levels of *Adra1* NAc core (A, B), *Adra2a* NAc core (C, D), *Adra2a*/*Adra1* NAc core (E, F), and *Adra2a* NAc core/shell (G, H) during cocaine and heroin (A, C, E, G) or sucrose (B, D, F, H) wd. Individual values are presented as well as the mean ± SEM. Differences relative to the control group, *p<0.05, **p<0.01, ***p<0.001.

Figure 3. Changes in gene expression of adrenoceptors in the dorsomedial prefrontal cortex during withdrawal. Levels of *Adra1* (A, B), *Adra2a* (C, D), and *Adrb1* (E, F) during cocaine and heroin (A, C, E) or sucrose (B, D, F) wd. Individual values are presented as well as the mean ± SEM. Differences relative to the control group, *p<0.05, **p<0.01, ***p<0.001; differences relative to the same treatment on different wd days, *f* p<0.05, *ff* p<0.01, *fff* p<0.001; general differences between the two wd days, Δ p<0.05, ΔΔ p<0.01, ΔΔΔ p<0.001.

Figure 4. Changes in gene expression of adrenoceptors in the dorsomedial prefrontal cortex (and in relation with BLA) during withdrawal. Levels of *Adra2a*/*Adra1* dmPFC (A, B), *Adra2a*/*Adrb1* dmPFC (C, D), *Adra1*/*Adrb1* dmPFC (E, F), *Adra1* dmPFC/vmPFC (G, H), and *Adra2a* BLA/dmPFC (I, J) during cocaine and heroin (A, C, E, G, I) or sucrose (B, D, F, H, J) wd. Individual values are presented as well as the mean ± SEM. Differences relative to the control group, *p<0.05, **p<0.01, ***p<0.001; differences relative to the same treatment on different wd days, *f* p<0.05, *ff* p<0.01, *fff* p<0.001; general differences between the two wd days, Δ p<0.05, ΔΔ p<0.01, ΔΔΔ p<0.001.

Figure 5. Heatmap of correlations between different parameters. For each pair of variables that experienced changes, we report the r value of the correlation for each experimental group. A bold value indicates a significant r value; a boxed value indicates a significant change relative to their control groups (control treatment and abstinence day).

Figure 6. General scheme summarizing the perdurable (A, D), early (B, E), and late (C, F) effects in central (A-C) and peripheral (D-F) parameters after the incubation of cocaine (red), heroin (green), and sucrose (yellow) seeking. Individual molecules are depicted with a circle; ratios are depicted with a double headed arrow linking both parameters; correlations are depicted with a line connected both parameters. A grey line indicates a direct metabolic pathway between metabolites (Q: L-glutamine; E: L-glutamate; P: L-proline; O: L-ornithine). Abbreviations: dmPFC: dorsomedial prefrontal cortex; vmPFC: ventromedial prefrontal cortex; BLA: basolateral amygdala; CeA: central nucleus of the amygdala.

Tables 1 and 2. Physiological, biochemical, and neuromolecular profiles during withdrawal in rats exposed to saline, cocaine, or heroin (Table 1) and sucrose or water (Table 2). Data represent descriptive and inferential statistics for body and adrenal weights, plasma markers (corticosterone and amino acids), and mRNA expression of stress-related genes (e.g., *Crh*, *Crhr2*) and adrenergic receptors (*Adra1*, *Adra2a*, *Adrb1*), including their ratios, as well as protein expression for specific targets (CRH and CRHR2), across distinct brain regions: nucleus accumbens (shell and core), prefrontal cortex (dmPFC and vmPFC), and amygdala (BLA and Central Amygdala -CeA). Rats were evaluated at two time points: 1 day (wd1) and 30 days (wd30) of withdrawal. Descriptive statistics are presented as mean ± standard deviation (SD) for n = 6-8 per group. Inferential analysis was performed using a two-way ANOVA (drug x wd period), with main effects and interactions reported as F-statistics, p-values, and partial eta-squared (η^2p) as a measure of effect size. Post-hoc (REGWQ) comparisons were conducted to identify significant differences between treatment groups (saline, cocaine, and heroin). Interactions were analysed by simple effects analysis with Sidak correction. An 'x' indicates missing data or data that did not comply with ANOVA assumptions after all possible data transformations. Statistical significance was set at $p < 0.05$.

Table 3. Correlation analysis and statistical comparison of gene expression relationships across brain regions. The table displays Pearson correlation coefficients (r) between mRNA expression or protein levels of stress-related genes and adrenergic receptors in specific brain regions (e.g., *Crhr2*, CHR2, CRH, *Adrb1*, *Adra2a* within the dmPFC, vmPFC, BLA, and NAcc core). Data are categorized by experimental group (Water, Sucrose, Saline, Cocaine, and Heroin) and withdrawal period (1 day [wd1] and 30 days [wd30]). For each correlation, the coefficient (r), p-value (p), and sample size (N) are provided. Statistical comparisons between correlation coefficients were performed using Fisher's r-to-Z transformation. The resulting Z-scores (Z) and corresponding p-values (p) indicate the significance of differences between withdrawal periods within the same treatment (e.g., wd1 vs. wd30) and between treatment groups at a specific time point (e.g., saline vs. cocaine). Statistical significance for both correlations and Z-tests was defined as $p < 0.05$.

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Figure 1

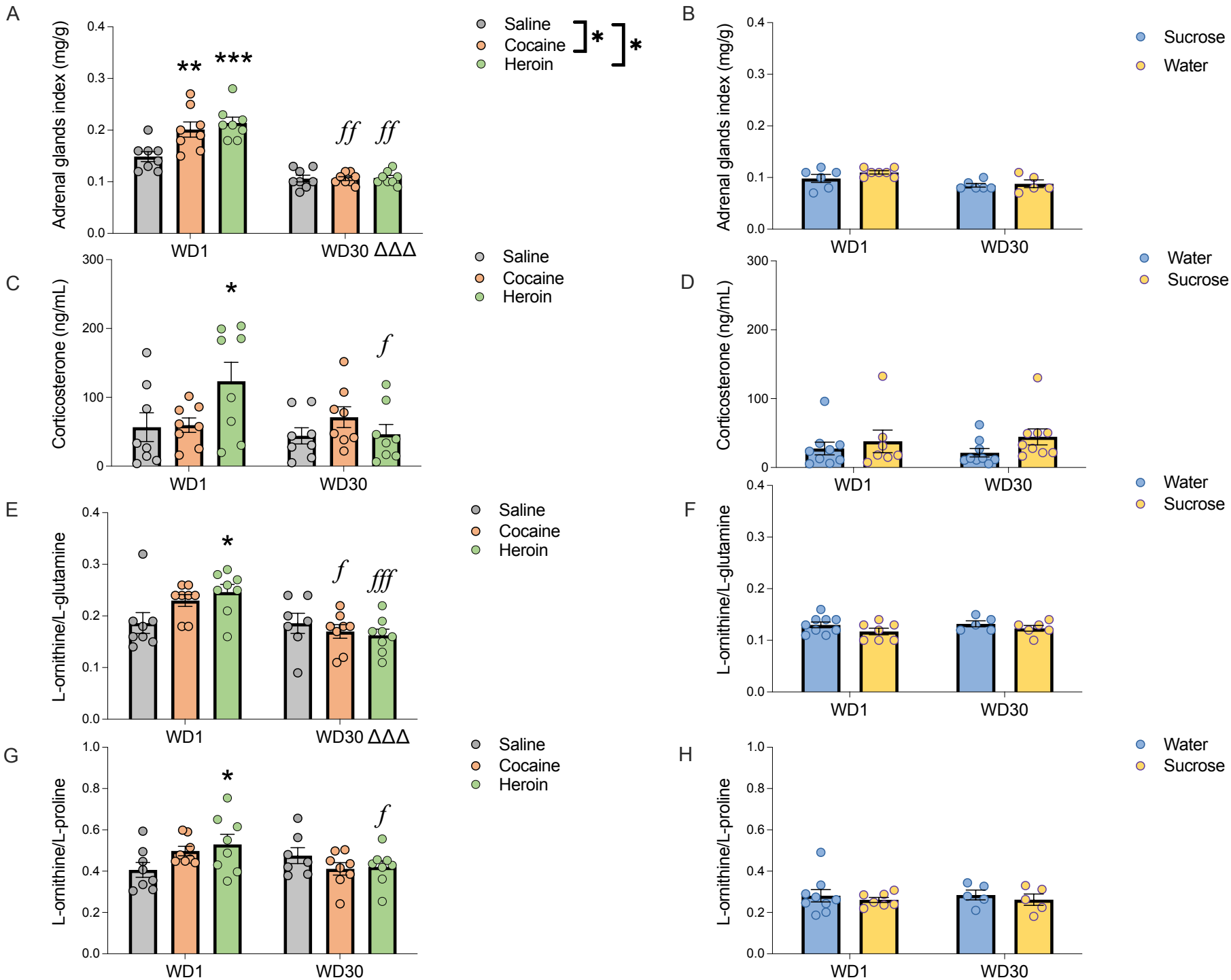
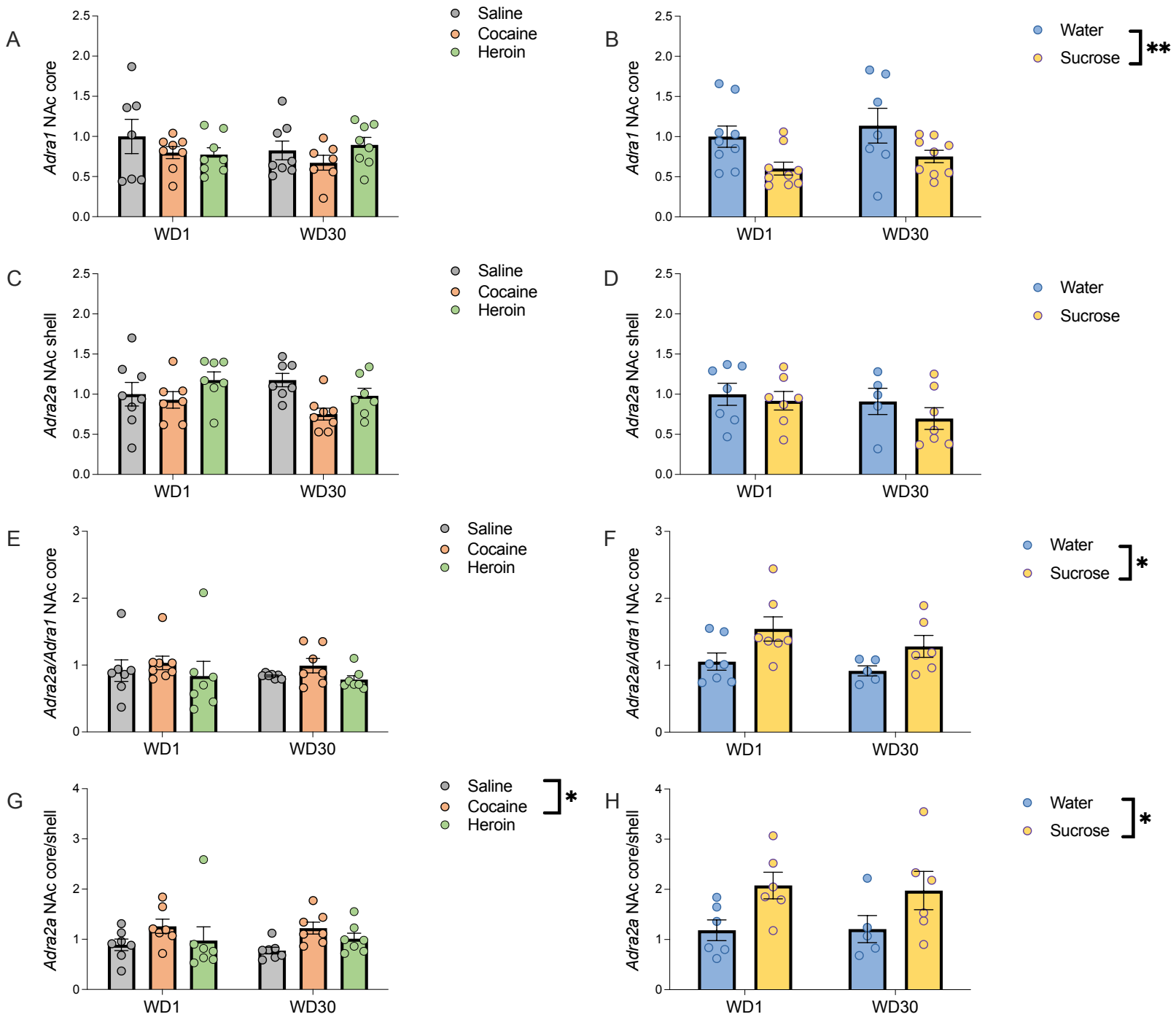

A
Adrenal glands index (mg/g)
0.4
0.3
0.2
0.1
0.0
**
***
ff
ff
WD1
WD30 ΔΔΔ
Saline
Cocaine
Heroin
*
*
B
Adrenal glands index (mg/g)
WD1
WD30
Sucrose
Water
C
Corticosterone (ng/mL)
300
200
100
0
*
f
WD1
WD30
Saline
Cocaine
Heroin
D
Corticosterone (ng/mL)
WD1
WD30
Water
Sucrose
E
L-ornithine/L-glutamine
*
f
fff
WD1
WD30 ΔΔΔ
Saline
Cocaine
Heroin
F
L-ornithine/L-glutamine
WD1
WD30
Water
Sucrose
G
L-ornithine/L-proline
1.0
0.8
0.6
0.4
0.2
0.0
*
f
WD1
WD30
Saline
Cocaine
Heroin
H
L-ornithine/L-proline
WD1
WD30
Water
Sucrose

Figure 2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Adra1 NAc core
2.5
2.0
1.5
1.0
0.5
0.0
WD1
WD30
Saline
Cocaine
Heroin
B
Adra1 NAc core
Water
Sucrose
**
C
Adra2a NAc shell
Saline
Cocaine
Heroin
D
Adra2a NAc shell
Water
Sucrose
E
Adra2a/Adra1 NAc core
3
2
1
0
Saline
Cocaine
Heroin
F
Adra2a/Adra1 NAc core
Water
Sucrose
*
G
Adra2a NAc core/shell
4
3
2
1
0
Saline
Cocaine
Heroin
*
H
Adra2a NAc core/shell
Water
Sucrose
*

Figure 3

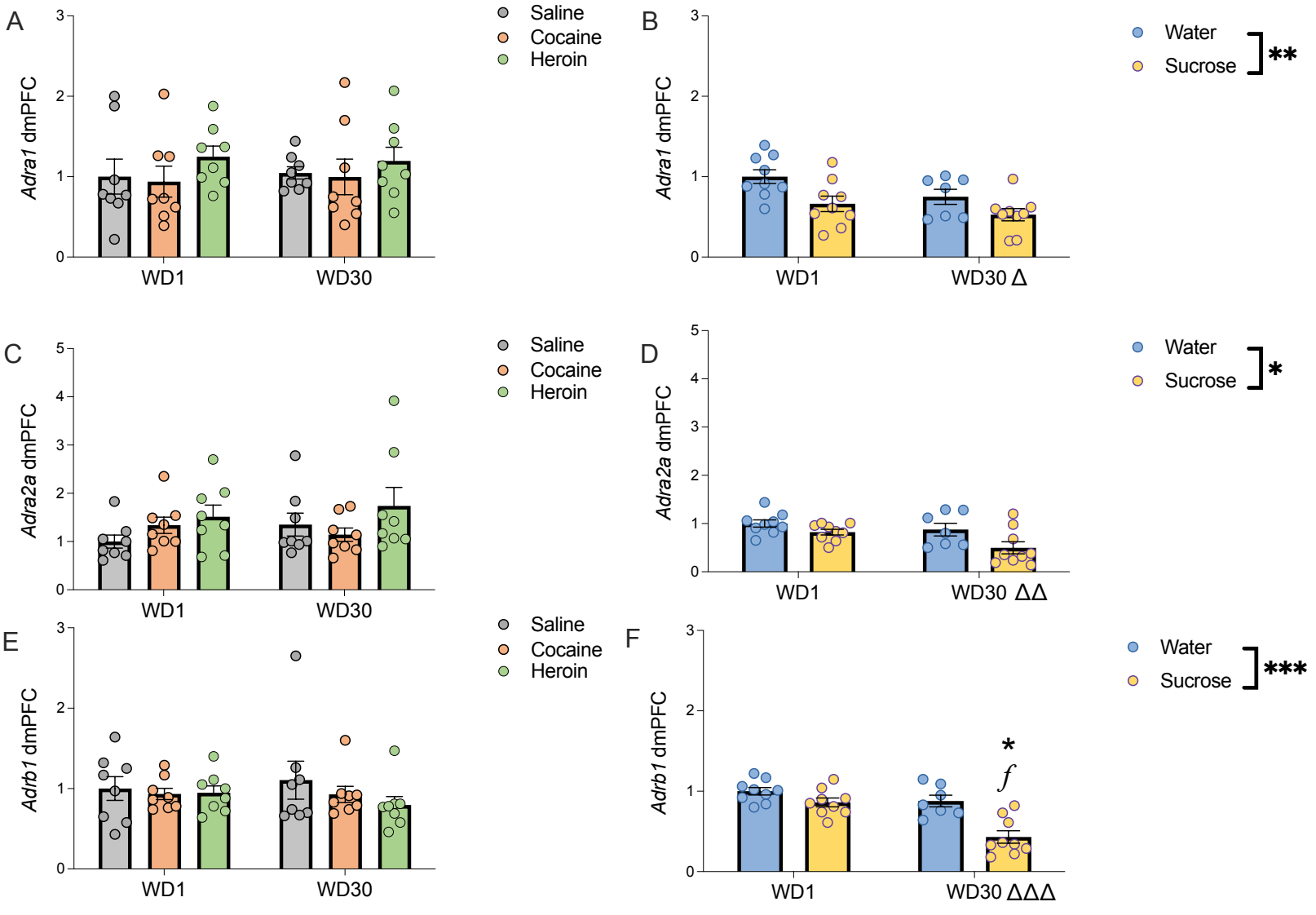

A
Adra1 dmPFC
WD1
WD30
Saline
Cocaine
Heroin
B
Adra1 dmPFC
WD1
WD30 Δ
Water
Sucrose
**
C
Adra2a dmPFC
WD1
WD30
Saline
Cocaine
Heroin
D
Adra2a dmPFC
WD1
WD30 ΔΔ
Water
Sucrose
*
E
Adrb1 dmPFC
WD1
WD30
Saline
Cocaine
Heroin
F
Adrb1 dmPFC
WD1
WD30 ΔΔΔ
*
f
Water
Sucrose
***

Figure 4

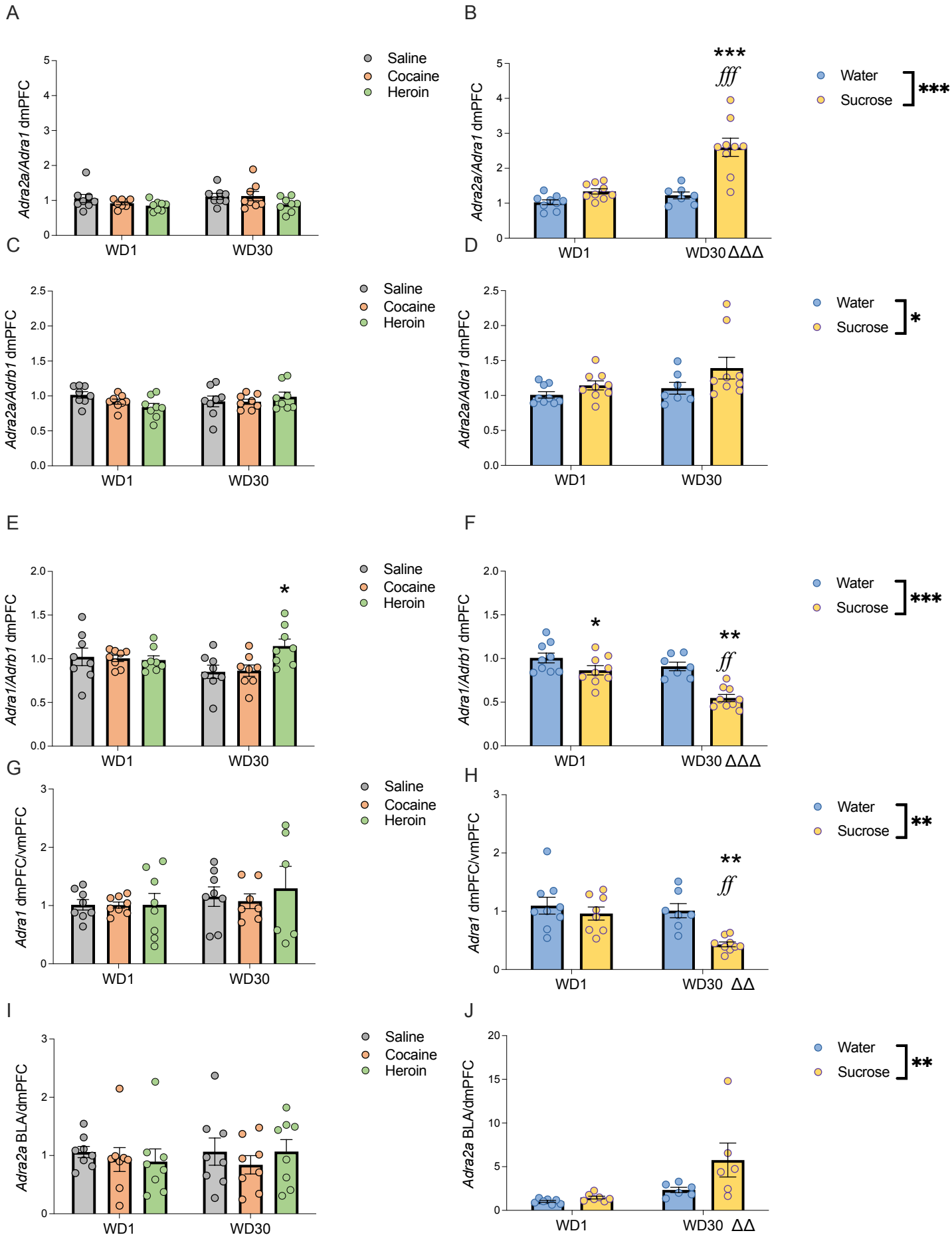
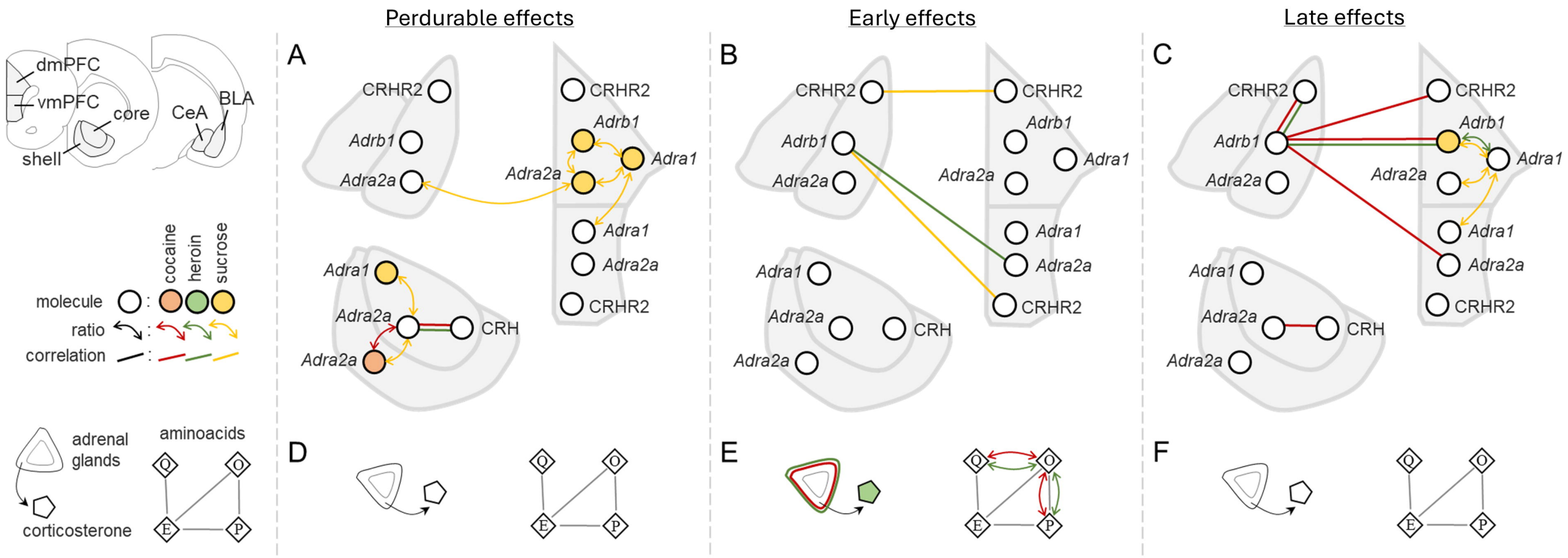

A
Adra2a/Adra1 dmPFC
Saline
Cocaine
Heroin
WD1
WD30
B
Adra2a/Adra1 dmPFC
***
fff
Water
Sucrose
***
WD1
WD30 ΔΔΔ
C
Adra2a/Adrb1 dmPFC
Saline
Cocaine
Heroin
WD1
WD30
D
Adra2a/Adra1 dmPFC
Water
Sucrose
*
WD1
WD30
E
Adra1/Adrb1 dmPFC
*
Saline
Cocaine
Heroin
WD1
WD30
F
Adra1/Adrb1 dmPFC
*
**
ff
Water
Sucrose
***
WD1
WD30 ΔΔΔ
G
Adra1 dmPFC/vmPFC
Saline
Cocaine
Heroin
WD1
WD30
H
Adra1 dmPFC/vmPFC
**
ff
Water
Sucrose
**
WD1
WD30 ΔΔ
I
Adra2a BLA/dmPFC
Saline
Cocaine
Heroin
WD1
WD30
J
Adra2a BLA/dmPFC
Water
Sucrose
**
WD1
WD30 ΔΔ

Figure 5

	wd1			wd30			wd1		wd30	
	saline	cocaine	heroin	saline	cocaine	heroin	water	sucrose	water	sucrose
Adrb1 BLA x CRHR2 vmPFC	0,233	0,069	0,466	0,059	0,474	0,176	0,107	**0,907**	0,153	-0,500
Adrb1 BLA x CRHR2 dmPFC	0,041	-0,375	-0,434	0,001	**0,859**	0,262	0,398	-0,009	0,477	-0,234
Adrb1 BLA x CRHR2 BLA	-0,147	-0,432	-0,212	0,245	**0,944**	**-0,905**	-0,267	0,054	0,001	-0,554
Adrb1 BLA x *Adrb1* dmPFC	0,182	0,628	-0,198	0,166	**-0,939**	**0,696**	0,139	-0,367	0,711	-0,309
Adrb1 BLA x *Adra2a* vmPFC	-0,524	0,281	**-0,642**	0,290	**-0,753**	0,323	0,067	-0,205	-0,342	0,141
CRHR2 BLA x CRHR2 dmPFC	0,235	**0,873**	0,339	0,110	**0,826**	0,029	-0,210	**0,915**	0,244	0,321
CRH core x *Adra2a* core	**-0,949**	-0,122	-0,202	**-0,779**	**0,771**	0,208	0,809	0,378	-0,175	-0,122

Figure 6


Perdurable effects
Early effects
Late effects
dmPFC
vmPFC
core
shell
CeA
BLA
cocaine
heroin
sucrose
molecule
ratio
correlation
A
B
C
CRHR2
Adrb1
Adra2a
Adra1
CRH
D
E
F
adrenal glands
corticosterone
aminoacids
Q
O
E
P