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Central nucleus of the amygdala as a common substrate of the incubation of drug and natural reinforcer seeking

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Abstract

Relapse into drug use is a major problem faced by recovering addicts. In humans, an intensification of the desire for the drug induced by environmental cues—incubation of drug craving—has been observed. In rodents, this phenomenon has been modeled by studying drug seeking under extinction after different times of drug withdrawal (or using a natural reinforcer). Although much progress has been made, an integrated approach simultaneously studying different drug classes and natural reward and examining different brain regions is lacking. Lewis rats were used to study the effects of cocaine, heroin, and sucrose seeking incubation on six key brain regions: the nucleus accumbens shell/core, central/basolateral amygdala, and dorsomedial/ventromedial prefrontal cortex. We analyzed PSD95 and gephyrin protein levels, gene expression of glutamatergic, GABAergic and endocannabinoid elements, and amino acid transmitter levels. The relationships between the areas studied were examined by Structural Equation Modelling. Pathways from medial prefrontal cortex and basolateral complex of the amygdala to central nucleus of the amygdala, but not to the nucleus accumbens, were identified as common elements involved in the incubation phenomenon for different substances. These results suggest a key role for the central nucleus of amygdala and its cortical and amygdalar afferences in the incubation phenomenon, and we suggest that by virtue of its regulatory effects on glutamatergic and GABAergic dynamics within amygdalar circuits, the endocannabinoid system might be a potential target to develop medications that are effective in the context of relapse.

KEYWORDS

central nucleus of the amygdala, drugs of abuse, endocannabinoid system, incubation of seeking, natural rewards, nucleus accumbens

1 | INTRODUCTION

An important barrier that addicts have to overcome in their recovery process is the risk of relapse. Recent studies in humans show that cue-induced drug craving for nicotine, alcohol, and methamphetamine or physiological responses to cocaine cues increases during abstinence.[1-4] Animal models can reproduce this phenomenon,[5,6] allowing the neural changes that occur during abstinence and that promote the increase in drug seeking behavior seen after protracted withdrawal or abstinence to be explored.[7]

However, the studies performed so far have mostly focused on cocaine,[8] and little attention has been paid to the incubation of opiate craving or that of other drugs.[9-15] Moreover, most reports have focused on only a single brain region, commonly the nucleus accumbens (NAc), and very few studies have simultaneously analyzed the role of different brain pathways or networks in this phenomenon.[16-21]

In terms of the neurochemical systems involved, glutamatergic transmission (GluA2-lacking calcium-permeable AMPA receptors in nucleus accumbens) appears to be critical for cocaine seeking incubation.[22] However, this involvement is not clearly established for the incubation of seeking for all substances.[8] Although the glutamatergic system represents a potential target to develop pharmacological approaches aimed at achieving abstinence, direct agonism or antagonism of its elements is troublesome because of the potential side effects. For this reason, other strategies like positive allosteric modulation are being investigated.[23,24] Another way of indirectly modulating the glutamatergic (and GABAergic) system is through the endocannabinoid system. Endocannabinoids bind to their receptors in the brain (mostly CB1 receptors) and inhibit neurotransmitter release either through direct G protein-dependent inhibition of presynaptic Ca2+ influx through voltage-gated Ca2+ channels (short-term plasticity) or through inhibition of adenylyl cyclase and downregulation of the cAMP/PKA pathway (long-term plasticity).[25] Exposure to drugs of abuse modifies synaptic plasticity by mechanisms that involve endocannabinoid signaling. For example, administration of THC (or cannabinoid agonists) or cocaine enhances the burst firing of VTA dopamine neurons and stimulates phasic dopamine release in the NAc in a CB1-dependent manner—see Zlebnik and Cheer[26] for a review. Exposure to THC or cocaine also affects synaptic plasticity in the NAc.[27-30] However, in spite of all this evidence, no experiments have examined the role of this system in the incubation of seeking phenomenon.

In the light of the above, the main aim of this study was to gain a deeper understanding of the psychobiological mechanisms that could be cardinal to the incubation phenomenon, simultaneously studying different classes of drugs (a psychostimulant like cocaine and an opiate such as heroin) and a natural reinforcer like sucrose. We adopted an integrated approach by analyzing six brain regions thought to be important for the incubation phenomenon, obtaining measures of the glutamatergic, GABAergic, and endocannabinoid systems. These data were then subjected to a multivariate statistics analyses to study the coordinated changes in these parameters in the six brain areas studied. Our results suggest that the central nucleus of amygdala appears to be a common hub underlying the phenomenon of incubation of seeking.

2 | MATERIALS AND METHODS

2.1 | Animals

We used male Lewis rats purchased from Harlan International Ibérica S.A. (*N* = 109) that weighed 300 to 320 g at the beginning of the experiments. Upon arrival, the rats were housed in groups of three in the vivarium at a constant temperature (20 ± 2°C) and on a 12 hours:12 hours light:dark cycle (lights on at 08:00 AM), with food (Panlab, commercial diet for rodents A04/A03) and water available ad libitum. Rats were maintained and handled in accordance with European Union Laboratory Animal Care Standards (2010/63/EU).

2.2 | Experimental design

We performed two experiments; the first one was aimed to reproduce the incubation phenomenon in Lewis rats as it was unknown if they would exhibit incubation (experiment 1), and the second one was performed to obtain brain tissue (Experiment 2). Three groups of rats that self-administered cocaine (n = 10), heroin (n = 16), or sucrose (n = 15) were studied in Experiment 1 and were then withdrawn from the drug (or sucrose) for one (wd1) and/or 30 days (wd30). After the withdrawal period, the rats were subjected to an extinction test in order to measure their seeking behavior. Two batches of rats were used in Experiment 2, one submitted to jugular catheter surgery (for drug or saline self-administration; see Data S1 for surgical procedures) and the other left intact (sucrose/water self-administration). After drug/saline or sucrose/water self-administration, the rats underwent 1 or 30 days of forced withdrawal (with regular handling) but without an extinction test (to avoid nonspecific effects from extinction learning or motor performance). The rats that received intravenous administration of cocaine, heroin, or saline (controls) were segregated into six groups (n = 8 rats per group, three substances × 2 withdrawal periods), while the rats that received oral sucrose or water were distributed in four groups (n = 9 rats per group, two substances × 2 withdrawal periods).

2.3 | Self-administration

All the self-administration sessions were performed in Skinner boxes (Coulbourn Instruments or Med-Associates), and they were monitored with Med-PC software (see Data S1 for behavioral data). The house light was off during the sessions, although we allowed some environmental light from the room (the door of the sound-attenuating cubicle was left ajar) so that the light:dark cycle of the rats was not altered. Two levers were used, an active and inactive lever. Each time the active lever was pressed by the rat (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 also switched on for 10 seconds at the same time. Subsequently, there was a time-out period of 40 seconds in which there were no programmed consequences, although the responses at each lever were recorded. Cocaine, heroin, or saline self-administration sessions lasted 6 hours each day, as described previously.[9,31] Rats orally self-administering sucrose or water were subjected to shorter sessions (2 h/d; Harkness, Webb, & Grimm[32]). 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 Vitulia-ERN) or tap water. In the first two self-administration sessions,

two sucrose pellets were placed on the active lever to facilitate the acquisition of self-administration behavior.

2.4 | Extinction test (experiment 1)

The extinction tests were identical to the self-administration sessions except that they lasted 3 hours in the case of cocaine and heroin, or 2 hours in the case of sucrose, and that the active lever press switched on the cue light but not the pump.

2.5 | Animal sacrifice

One day or one month after the last self-administration session, the rats from experiment 2 were weighed and sacrificed by decapitation between 11:00 and 13:00 AM. The rat's brain was extracted and submerged in isopentane chilled on dry ice for 10 seconds and stored at −70°C.

2.6 | Dissection and sample processing

Each brain was dissected using a cryostat according to the Paxinos atlas,[33] see Figure S1, and the tissue was stored at −70°C. Samples were homogenized in an RNase-free isosmolar buffer, and different aliquots were properly stored for subsequent analyses (see Data S1 for sample processing).

2.7 | Western blot

The glutamatergic and GABAergic anchoring proteins PSD95 and gephyrin were analyzed by western blot using the primary anti-PSD95 (Cell Signaling #2507) and anti-gephyrin (Cell Signaling #14304) antibodies. Proteins were analyzed by densitometry using ImageJ free software, and the data were normalized according to the criterion described previously[34] (see Data S1 for more details).

2.8 | Gene expression analysis by qPCR

RNA was isolated using a protocol adapted from the method described by Chomczynski and Sacchi[35] then treated with DNaseI and retrotranscribed. Different genes (NMDA receptor subunits 1, 2A, and 2B: *Grin1*, *Grin2a*, *Grin2b*; AMPA receptor subunits 1 and 2: *Gria1*, *Gria2*; $GABA_A$ receptor subunits alpha 1 and 2, gamma 2 and delta: *Gabra1*, *Gabra2*, *Gabrg2*, *Gabrd*; cannabinoid receptor 1, *Cnr1*, *N*-acylphosphatidylethanolamine phospholipase D, *Napepld*, fatty acid amidohydrolase, *Faah*, diacylglycerol lipase alpha, *Dagla* and monoacylglycerol lipase, *Mgll*) were analyzed by qPCR. The fold change value was calculated by the method described by Pfaffl,[36] using *Gapdh* as housekeeping gene[37] (see Data S1 for more details).

2.9 | Capillary electrophoresis

When there was sufficient tissue from the samples, the amine content was analyzed by capillary electrophoresis (L-glutamic acid, L-aspartic acid, glycine, L-glutamine, taurine, L-serine, D-serine, γ-aminobutyric acid) using a protocol adapted from a method previously described[38] (see Data S1 for more details).

2.10 | Univariant statistical analyses

To analyze the incubation of seeking behavior, we used a one-tailed unpaired Student's (parametric data) or Welch's (nonparametric data) *t* test to compare the number of total active lever responses during the extinction test between wd1 and wd30 rats. We then conducted a factorial mixed ANOVA with repeated measures to compare the progression of the infusions (active lever presses) and inactive lever presses along sessions (within subject factors), between experiments and withdrawal days (between subject factors), substance by substance. We carried out two-way ANOVAs to compare specific behavioral or biochemical parameters between the groups due to the treatment and/or withdrawal, followed by a simple effects analysis. Following a significant effect of the treatment, we performed the Ryan post hoc procedure (REGWQ) to allocate the differences between saline, cocaine, and heroin rats (Field[39]).

2.11 | Structural equation modeling

We wanted to study how the net balance between excitatory and inhibitory information (using PSD95/gephyrin ratio) was correlated between connected brain areas, and importantly, how these correlations changed during the treatment and withdrawal periods. These changes might suggest a coordinated activity in these areas, either directly or indirectly (via indirect or spurious connections). As a multivariate analysis, structural equation modeling (SEM) allows us to study all these correlations at the same time, making it possible to find differences in the correlations even if no differences were seen in the univariate analyses of the data.[40] We carried out a path analysis with maximum likelihood estimation (MLE) on SPSS Amos 22 software (IBM). The input variable was the PSD95/gephyrin ratio in all the six limbic regions analyzed, a measure of the excitatory/inhibitory synapse balance (Figure 1A). The model was composed of known connections (correlations) from glutamatergic (basolateral amygdala [BLA], dorsomedial prefrontal cortex [dmPFC], ventromedial PFC [vmPFC]) to GABAergic (NAc core and shell, and CeA) regions, and the covariances (CVs) between the glutamatergic regions, and between the NAc core and shell regions (see Data S1 for model design). To compare the strength of each path (that is, the value of its standardized regression weight-SRW) between groups, an unconstrained model was compared with a model in which this path (one-by-one, $\Delta df = 1$) had the same value for the two groups when the $\Delta\chi^2$ method was used (constrained model). When testing small sample sizes, as was our case (n = 7-9 rats per group), estimates of goodness of fit are not sufficiently accurate, yet group comparisons are still accurate when using anatomically established connections.[41] Nevertheless, we first checked the model with different combinations of samples in order to verify it with a sample size big enough (see Data S1 for model validation).

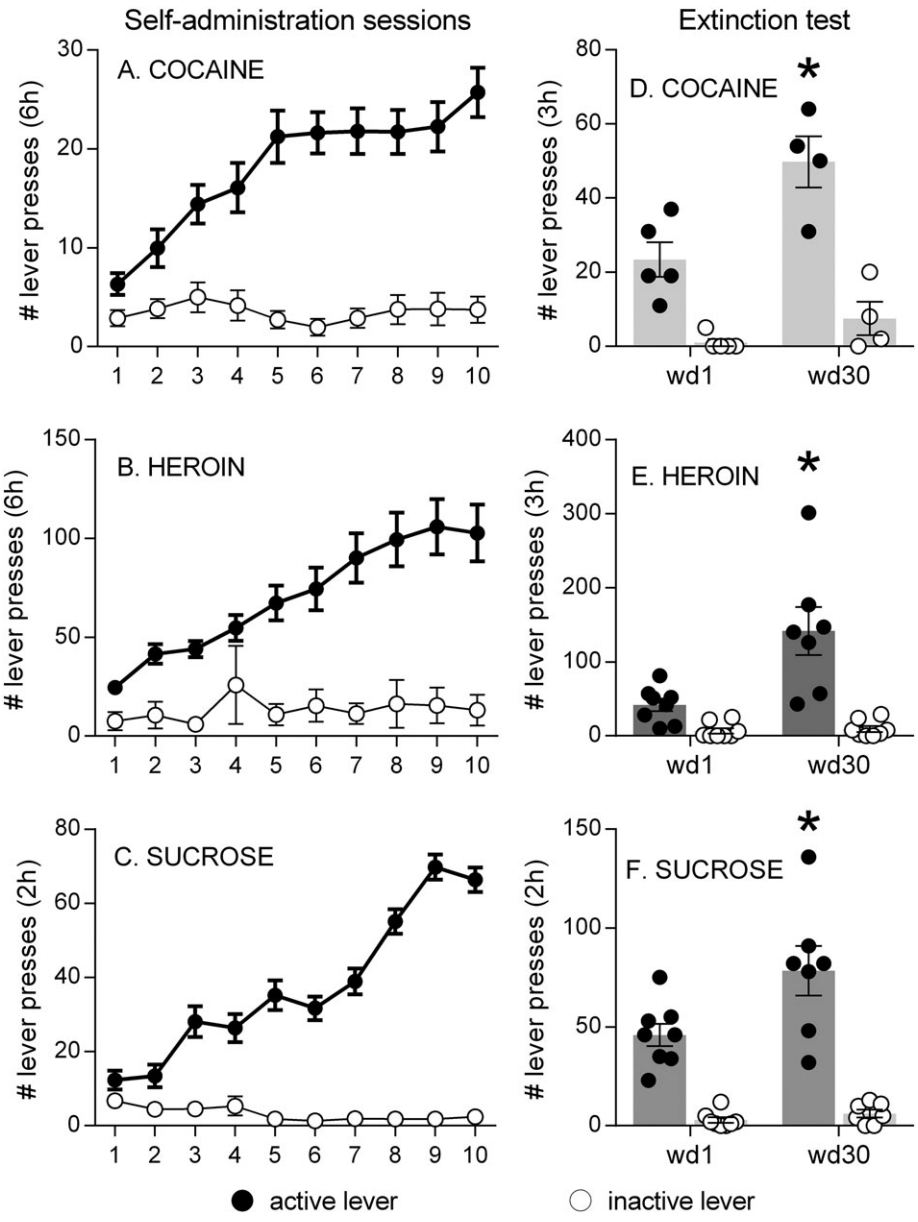

FIGURE 1 Self-administration behavior and extinction tests. Active (black circles) and inactive (white circles) lever press responses during 6 hours (cocaine, heroin) or 2 hours (sucrose) daily self-administration sessions (fixed ratio 1) and 3 hours (cocaine, heroin) or 2 hours (sucrose) extinction tests. A, Cocaine (n = 25), B, heroin (n = 32), and C, sucrose (n = 33) self-administration sessions of the rats from experiments 1 and 2. D, Cocaine (wd1, n = 5; wd30, n = 4), E, heroin (wd1, n = 8; wd30, n = 7), and F, sucrose (wd1, n = 8; wd30, n = 7) extinction sessions. * indicates differences ($P < 0.05$) in active lever responses between 1 and 30 days of withdrawal. Data are expressed as mean ± SEM

3 | RESULTS

3.1 | Self-administration and incubation of seeking

The incubation phenomenon was evident in Lewis rats, as witnessed by the higher number of total active lever presses in the wd30 group during the extinction test relative to wd1 groups (cocaine – $t_7 = 3.27$, $P = 0.0068$, $d = 2.47$; heroin – $t_{8.02} = 2.669$, $P = 0.0142$, $d = 1.89$; sucrose – $t_{13} = 2.483$, $P = 0.0137$, $d = 1.38$: Figure 1D-F; Table S3B).

We then ran a second experiment on a separate batch of rats (experiment 2) to obtain tissue samples. We sacrificed half of the rats the day after the last self-administration session (wd1) and the other half 30 days after (wd30). Rats progressively self-administered larger amounts of cocaine, heroin, and sucrose as the training sessions progressed (Figure 1A-C; significant lever effect for cocaine, heroin, and sucrose, $P < 0.001$; significant session effect for cocaine, heroin, and sucrose, $P < 0.01$, significant linear contrast for cocaine, heroin, and sucrose, $P < 0.0001$; significant lever × session interaction for cocaine, heroin, and sucrose, $P < 0.05$). Importantly, self-administration patterns were undistinguishable between batches ($P > 0.35$). Moreover, there were no differences in the self-administration patterns between rats from the wd1 and wd30 groups ($P > 0.18$) nor was there any interaction between the experiments and

the withdrawal time in the self-administration behavior ($P > 0.29$: Figure S4A-C, G-K; Table S3A).

3.2 | Integrated analysis of the synaptic scaffolding proteins PSD-95 and gephyrin

We performed an integrated analysis of the coordinated excitatory activity in six key regions of the brain. We fed the model with the PSD95/gephyrin ratio, the major proteins at the postsynaptic densities in excitatory and inhibitory synapses, respectively. This ratio was taken as an index of the net excitatory activity in a given region (Keith[42]; Yu & Blas[43]: see Tables S14 and S15 for values and S16 for comparisons).

By analyzing the SRWs, which are indicative of the strength of the paths in our model (for $\Delta\chi^2$ values see Table S16), after incubation of cocaine, heroin, or sucrose seeking (Figure 2), we found that the CeA was a nucleus with a common role in the incubation of seeking and more importantly, in both drugs and sucrose. Indeed, we observed a change in the direction (from negatively to positively correlated) of the strength of the path from BLA to CeA after a 30 days of withdrawal from cocaine or heroin relative to the first day (Figure 2I). There was also a functional disconnection between the dmPFC and CeA after incubation of heroin seeking, as reflected by the loss of the negative correlation on wd1 in heroin self-administering rats as compared to their corresponding saline self-administering controls. In addition, we also found that after the incubation of sucrose seeking, a near significant value of SRW in the control group is lost in sucrose abstinent rats (Figure 2G). With regard to the connections between the vmPFC and CeA, there are opposing results after heroin and cocaine seeking incubation, this path becoming strengthened or weakened, respectively (Figure 2H).

Contrary to CeA, no common effects were seen in any path to NAc. Connections from the vmPFC to the NAc core seem to be intensified after 30 days of forced heroin abstinence (Figure 2E) and those from the dmPFC debilitated after sucrose abstinence (Figure 2D).

Finally, we observed strengthening of the paths from dmPFC to NAc core (Figure 2D) and from BLA to NAc shell (Figure 2C) which we suggest are perdurable effects of heroin and cocaine self-administration, respectively.

3.3 | Levels of amino acid neurotransmitters and other brain amines

The incubation of cocaine and heroin seeking was associated with a decrease in the glutamate/glutamine ratio in the CeA, indicating lower glutamate turnover. In the case of cocaine, this decrement was preceded by an elevated ratio in wd1 rats (see Figure 3C). Another interesting incubation-related effect was the enhanced glutamate/D-serine ratio in the dmPFC of wd30 heroin-withdrawn rats (Figure 3B). Likewise, there was an increase in glutamate content in the vmPFC after incubation of cocaine seeking (Figure 3A).

With regard to sucrose seeking incubation, there was a normalization of a decreased glutamate/GABA ratio in the BLA (another indirect indication of the net excitatory activity; Figure 3D) and of elevated taurine levels in the NAc shell (Figure 3E). A decrease in L-aspartate in the NAc core was evident after 30 days of sucrose withdrawal (Figure 3F). See Table 1 for the parameters of ANOVAs.

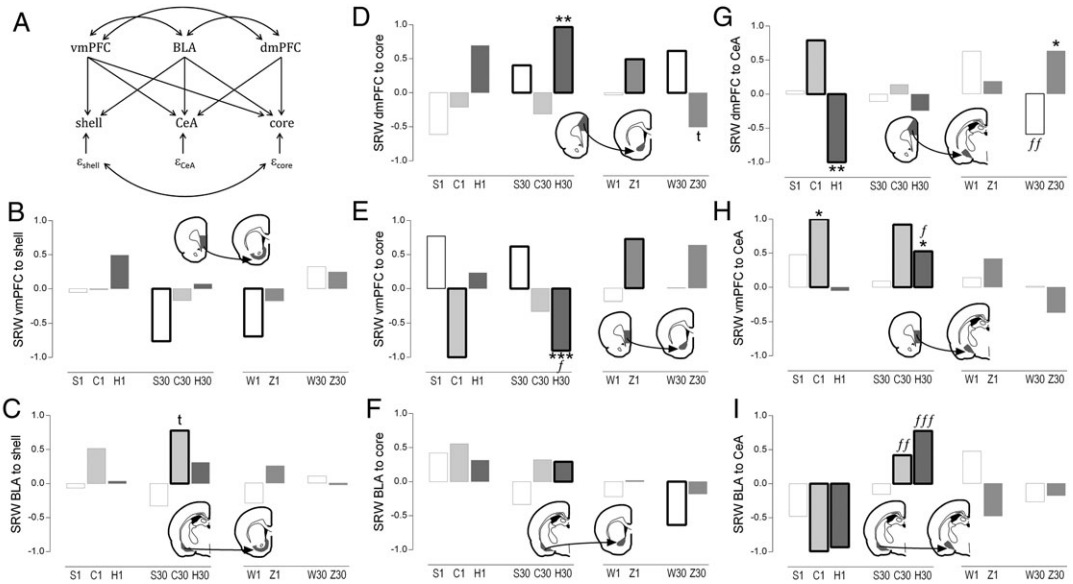

FIGURE 2 Structural equation modeling group comparisons. A, Model used in the study in which the vmPFC, dmPFC, and BLA PSD95/gephyrin ratios, as well as errors in the NAc shell and core ratios (ε), are the external variables, and the NAc shell, core, and CeA ratios are the internal variables. Regressions are represented as one-headed arrows and covariances as double-headed arrows. B-I, Value for the squared regression weight (SRW) for each path in each group, and the differences between the groups: B-C, paths to shell; D-F, paths to core; G-I, paths to CeA. D,G, paths from dmPFC; B,E,H, paths from vmPFC; C,F,I, paths from BLA. S, saline; C, cocaine; H, heroin; W, water; Z, sucrose; 1, wd1; 30, wd30. Border of the bars: grey and thin, SRW $P > 0.07$; black and thin, SRW $P < 0.07$; black and thick, SRW $P < 0.05$. Differences with the control group on the same withdrawal day: t $P < 0.054$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Differences with the same treatment on the different withdrawal days: *f* $P < 0.05$, *ff* $P < 0.01$, *fff* $P < 0.001$

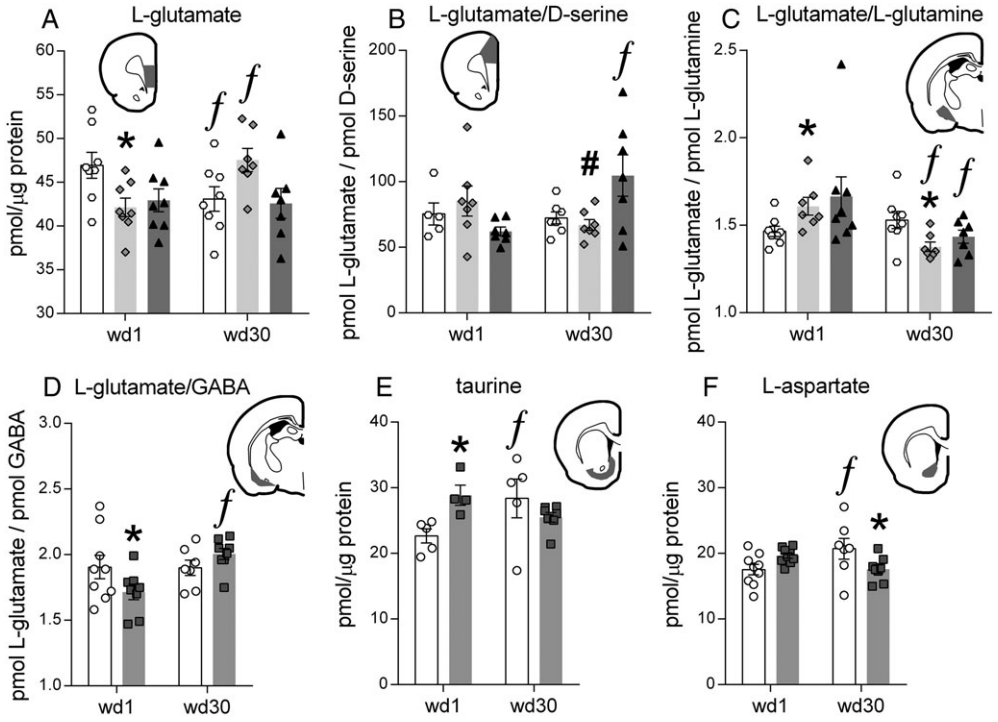

FIGURE 3 Changes in amine content during the incubation of seeking. Changes in A, the vmPFC , B, dmPFC, and C, CeA during cocaine and heroin withdrawal: White hexagons, saline; grey diamonds, cocaine; grey triangles, heroin. Changes in D, the basolateral amygdala, E, NAc shell, and F, core during sucrose withdrawal: White circles, water; grey squares, sucrose. Individual values are presented, as well as the mean ± SEM: Differences relative to the control group on the same withdrawal day, $^*P < 0.05$; differences relative to the same treatment on different withdrawal days, *f* $P < 0.05$; differences on the same day between the cocaine and heroin group, $\#P < 0.05$

3.4 | Expression of genes involved in the endocannabinoid system and of glutamatergic and GABAergic receptor subunits

The incubation of cocaine and heroin seeking was differentially associated with two patterns of changes. The first one consisted on increments in some parameters early in withdrawal, followed by a normalization 1 month later. The other pattern was associated to changes that appeared later on, during protracted abstinence.

Among the changes observed after heroin self-administration, we found an increase in the *Gabra1*/*Gabra2* ratio in the BLA (Figure 4A) and in *Actb* expression in the NAc core (Figure 4E). After cocaine self-administration, there was an evident increase in *Gria1* expression in the BLA (Figure 4A) and in the dmPFC (Figure 4C), accompanied by a slight increase in *Grin2a* and *Gria2* expression in the BLA (Figure 4A) and of the *Gria1*/*Gria2* ratio in the dmPFC (Figure 4C). Cocaine self-administration also induced an increase in the *Gabrg2*/*Gabrd* ratio in the NAc shell (Figure 4D), resulting from changes in the gene expression of these two $GABA_A$ subunits. Given that the different subunits of ionotropic receptors dictate the properties, location, and kinetics of ion channels, the differences that we observe after cocaine self-administration are likely to be associated with altered synaptic activities.

The expression of genes related to the endocannabinoid system was also affected after protracted abstinence. A common decrease in *Napepld*/*Faah* ratio was observed in the BLA both after cocaine and heroin self-administration along with a concomitant increase in *Faah* expression in wd30 heroin rats (Figure 4A). A decrement of *Napepld* expression was also evident after cocaine abstinence in NAc shell (Figure 4D). Since *Napepld* and *Faah* are the main synthesis and eliminating enzymes in anandamide (AEA) metabolism, it is tempting to speculate that the synthesis of this molecule may be decreased after drug-seeking incubation in these regions. After 30 days of withdrawal from sucrose, the gene expression of the degrading enzyme (*Mgll*) of 2-arachydonoylglycerol (2-AG) was elevated in the CeA (Figure 5B), while heroin seeking incubation was associated to an elevation of the gene expression of the synthesis enzyme, *Dagla*, in the BLA (Figure 4A). In the view of the above-mentioned modifications, we suggest that altered levels of 2-AG in these regions may contribute to the incubation phenomenon.

The most notable change after protracted withdrawal from sucrose was a decrease in the level of several genes in dmPFC: *Actb*, *Grin1*, *Gria1*, *Gabra1*, and *Gabra2*, as well as in *Gabra1*/*Gabra2* ratio (Figure 5A). These changes could be reflective of impaired plasticity in this area, as suggested by the low levels of transcripts of actin cytoskeleton and of glutamatergic and GABAergic ionotropic receptors subunits. Minor alterations were also observed in mPFC after protracted drug withdrawal: an increase of *Gabrd* expression in vmPFC after heroin withdrawal (Figure 4B) and an augmented *Grin2a*/*Grin2b* ratio in dmPFC after cocaine withdrawal (Figure 4C). See Table 1 for ANOVA parameters.

TABLE 1 ANOVA of the changes in amine content and gene expression during the incubation of seeking

Substance	Region	Parameter	F (df_M,df_R)	P	η^2
Capillary electrophoresis					
Cocaine and heroin					
	CeA	L-glu/L-gln	$F_{2,38}$ = 7.105	0.002	0.272
Cocaine					
	vmPFC	L-glu	$F_{2,40}$ = 5.754	0.006	0.223
Heroin					
	dmPFC	L-glu/D-ser	$F_{2,34}$ = 5.349	0.010	0.239
Sucrose					
	BLA	L-glu/GABA	$F_{1,28}$ = 4.763	0.038	0.145
	Core	L-asp	$F_{1,29}$ = 8.729	0.006	0.231
	Shell	Tau	$F_{1,17}$ = 7.145	0.016	0.296
Gene expression					
Cocaine and heroin					
	BLA	*Napepld/Faah*	$F_{2,41}$ = 6.587	0.003	0.236
Cocaine					
	BLA	*Grin2a*	$F_{2,42}$ = 3.620	0.035	0.131
	BLA	*Gria1*	$F_{2,42}$ = 6.461	0.004	0.218
	BLA	*Gria2*	$F_{2,42}$ = 4.394	0.019	0.165
	dmPFC	*Grin2a/Grin2b*	$F_{2,42}$ = 4.649	0.015	0.158
	dmPFC	*Gria1*	$F_{2,42}$ = 3.310	0.046	0.131
	dmPFC	*Gria1/Gria2*	$F_{2,42}$ = 3.148	0.053	0.125
	Shell	*Gabrd*	$F_{2,38}$ = 3.575	0.038	0.150
	Shell	*Gabrg2*	$F_{2,38}$ = 3.195	0.052	0.130
	Shell	*Gabrg2/Gabrd*	$F_{2,36}$ = 6.903	0.003	0.262
	Shell	*Napepld*	$F_{2,38}$ = 3.727	0.033	0.124
Heroin					
	BLA	*Gabra1/Gabra2*	$F_{2,42}$ = 3.507	0.039	0.132
	BLA	*Dagla*	$F_{2,42}$ = 4.952	0.012	0.186
	BLA	*Faah*	$F_{2,41}$ = 3.621	0.036	0.144
	vmPFC	*Gabrd*	$F_{2,37}$ = 4.196	0.023	0.126
	Core	*Actb*	$F_{2,38}$ = 3.424	0.043	0.138

(Continues)

TABLE 1 (Continued)

Substance	Region	Parameter	F (df_M,df_R)	P	η^2
Sucrose					
	CeA	*Mgll*	$F_{1,29}$ = 5.253	0.029	0.133
	dmPFC	*Grin1*	$F_{1,30}$ = 4.717	0.038	0.080
	dmPFC	*Gria1*	$F_{1,30}$ = 4.128	0.051	0.064
	dmPFC	*Actb*	$F_{1,30}$ = 10.221	0.003	0.111
	dmPFC	*Gabra1*	$F_{1,30}$ = 4.054	0.053	0.083
	dmPFC	*Gabra2*	$F_{1,30}$ = 4.622	0.040	0.091
	dmPFC	*Gabra1/Gabra2*	$F_{1,30}$ = 4.861	0.035	0.092

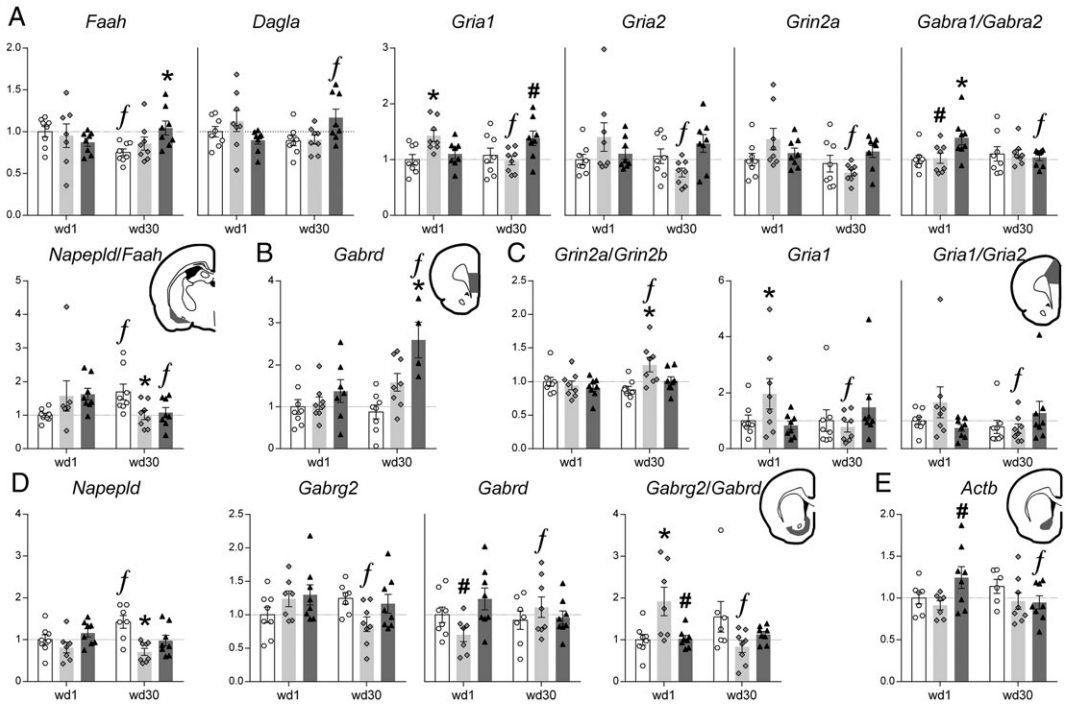

FIGURE 4 Changes in gene expression during the incubation of cocaine and heroin seeking. Changes in A, basolateral amygdala, B, vmPFC, C, dmPFC, D, NAc shell, and E, core: White hexagons, saline; grey diamonds, cocaine; grey triangles, heroin. Individual fold change values are presented, as well as the mean ± SEM: Differences relative to the control group on the same withdrawal day, *P < 0.05; differences relative to the same treatment on different withdrawal days, *f* P < 0.05; differences on the same day between the cocaine and heroin group, #P < 0.05

A subsequent multivariate analysis of gene expression using principal component analysis revealed differentially modulated clusters of genes in the studied regions, especially in the BLA during the incubation of heroin and cocaine seeking (see Figures S6 and S7 for a list of the factors identified and Tables S17 and S18 for treatment, withdrawal, and treatment × withdrawal effects).

Effects related to the different treatments without changes throughout the withdrawal were also observed. These changes in amine content and gene expression could be derived from self-administration but not related to the incubation phenomenon and are presented in the Data S1.

4 | DISCUSSION

We present here the first integrative analysis of the incubation of seeking phenomenon, comparing two types of drugs and a natural

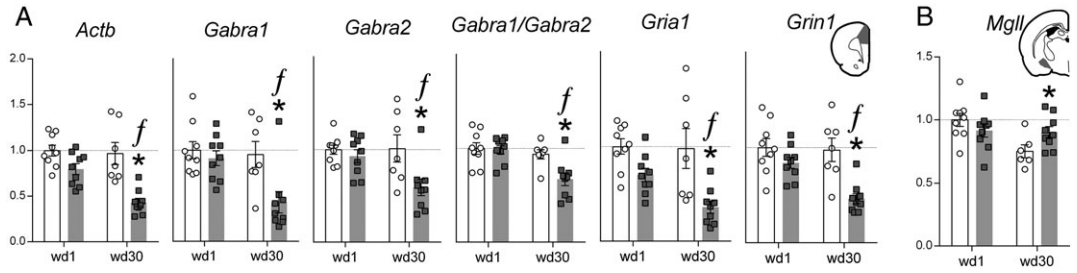

FIGURE 5 Changes in gene expression during the incubation of sucrose seeking. Changes in A, dmPFC and B, central nucleus of the amygdala: White circles, water; grey squares, sucrose. Individual fold change values are presented, as well as the mean ± SEM: Differences relative to the control group on the same withdrawal day, $^{*}P < 0.05$; differences relative to the same treatment on different withdrawal days, f $P < 0.05$

reinforcer in the same experiment, and analyzing the effects of such phenomenon on six different brain regions (the NAc [core and shell], the amygdala [basolateral complex and central division], and the medial prefrontal cortex [dorsal and ventral divisions]), as well as on two neurotransmitter (glutamate, GABA) and one neuromodulatory (endocannabinoid) system. Each of the substances that we chose to study (a psychostimulant, an opiate, and a natural reinforcer) may be exerting specific pharmacological actions on the circuits that regulate seeking, both at early and protracted stages of abstinence, leading to effects that may not necessarily be related to the incubation phenomenon. Keeping this limitation in mind, our approach was to focus on the common adaptations caused by these three substances and to look for neural loci that may be congregating these common alterations.

4.1 | The amygdala as a common hub of the incubation of seeking

Our main finding is that the central nucleus of amygdala is the final common hub that agglutinates the relevant afferents modulated by the incubation of heroin, cocaine, and sucrose seeking (Figure 6B-D). Thus, both drugs of abuse and the natural reinforcer elicited an impairment in neurotransmission homeostasis in CeA but by different mechanisms, depending on the nature of the reward. While incubation of drug (cocaine and heroin) seeking was associated to decreased glutamate turnover, incubation of sucrose seeking was associated with increased levels of *Mgll* expression (possibly leading to a decrease in the substrate of the enzyme, 2-AG). As recently published,[44] the inhibition of this enzyme in the CeA is able to reduce anxiety-like behavior and alcohol-consumption in alcohol-dependent rats, making it a promising target to reduce the development of the incubation of seeking. Given the complex internal structure of the CeA,[45] it is not clear how the imbalance in glutamate turnover or 2-AG levels translate into enhanced CeA activity. These results support the previous implication of this amygdalar nucleus in the incubation of cocaine,[46,47] methamphetamine,[48] morphine,[11] sucrose,[49] and nicotine seeking.[50]

Coincidently with the impairment in CeA activity, we observed a qualitative and quantitative alteration in the way BLA (cocaine and heroin) and dmPFC (heroin and sucrose) respectively communicate with CeA. The correlation of the net excitatory content in BLA with that of CeA moved from negative to positive after a month of withdrawal

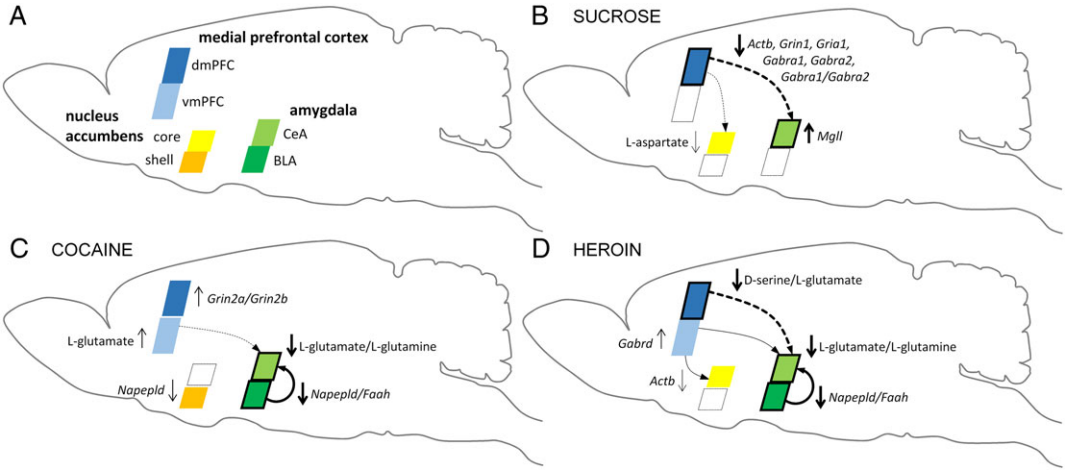

FIGURE 6 General scheme summarizing the neurobiological changes observed after the incubation of cocaine, heroin, and/or sucrose seeking. A, Regions are represented by the polygons. Paths analyzed in structural equation modelling (SEM) are depicted by the arrows. B-D, Changes during the incubation of sucrose, cocaine, and heroin seeking. Colored boxes represent regions where incubation of seeking provokes biochemical changes (in amine content or gene expression). Continuous arrows represent positive SWRs in SEM, while dashed arrows represent a loss of coherence. Thick arrows represent common SEM changes between two substances, and thin arrows represent changes specific to one of the substances. Bold words represent common biochemical changes between two substances

(Figure 6C,D). The meaning of this shift is not easy to interpret, yet it is tempting to invoke a role for the intercalated cell masses of the amygdala.[51] This shift was coincident with decreased anandamide levels in protracted withdrawal (as reflected by the *Napepld*/*Faah* ratio), preceded by changes in glutamatergic (cocaine) or GABAergic (heroin) systems in early withdrawal. Given that a decrement in AEA has been related with an activated BLA,[52] this alteration could represent a reactive state of this structure after incubation of seeking. As it seems that the basal region of the amygdala is not involved in the incubation of the seeking,[11,16,46] it is possible that the lateral amygdala could be the structure playing a role in the process.

4.2 | The prefrontal cortex and the incubation of seeking

Contrary to the BLA, there was a quantitative change in the dmPFC, showing no correlation with CeA after 1 month of heroin or sucrose withdrawal (Figure 6B,D). At the same time, we suggest that there could be an impaired plastic capacity in this region: First, protracted heroin abstinent rats showed a lower level of the NMDAR coagonist D-serine relative to glutamate levels, indicative of a potentially impaired plasticity as discussed by Curcio et al[53]; second, there was a severe depletion of genes (*Actb*, *Gabra1*, *Gabra2*, *Gria1*, *Grin1*) in the dmPFC of sucrose wd30 abstinent rats. We did not observe these alterations after 1 month of cocaine withdrawal. This is in accordance to the lack of implication of this cortical region in the incubation of psychostimulants seeking.[5,18,54] In a recent study using functional magnetic resonance with obese subjects trying two kinds of diet, researchers observed that the control exerted by the dorsolateral prefrontal cortex (dlPFC) in the presence of food-related cues over subcortical structures like the amygdala was heightened in the case of subjects on a diet that greatly reduced food cravings.[55] This research aligns with our results in sucrose seeking incubation since the rat mPFC is the equivalent cortical area of human dlPFC.[56] Regarding the vmPFC, no common changes were seen among the different substances, so it is possible that the observed effects were specific to each substance and not likely related with the incubation of seeking phenomenon.

If CeA is the main center of incubation, which of its proposed functions is responsible for initiating or maintaining the phenomenon? This region of amygdala is known to exert its influence over neuromodulatory systems, like dopaminergic, serotonergic, and noradrenergic systems. Its role in the incubation of seeking could explain why the inhibition of dopamine receptors in dorsomedial striatum prevents the expression of the incubation of methamphetamine seeking,[57] or why systemic D1 antagonism reduces sucrose seeking incubation.[58] Furthermore, it could also explain why β-endorphin increase during early cocaine withdrawal extinction test is lost weeks later.[59] The same reasoning can also be applied to the relationship between 5HT2C receptors in the mPFC and the incubation of cocaine seeking.[60]

4.3 | Changes in the nucleus accumbens during the incubation of seeking

We failed to find common changes occurring in the nucleus accumbens. This is not surprising since there is evidence that this region is not involved in the incubation of seeking of a natural reinforcer like sucrose. It has been shown that the manipulations of the core and shell subregions of the accumbens at early and protracted cocaine abstinence are able to modify incubated responses.[16,31,61-63] Nevertheless, using a protocol of self-administration that promotes incubation of sucrose seeking, Grimm et al[58] showed that an inhibitor of dopamine D1 receptor delivered into core or shell regions of the nucleus accumbens, both during early or protracted abstinence, equally reduced seeking responses. No common biochemical changes or cue-induced effects were seen either after the incubation of cocaine and sucrose seeking. For example, after cocaine seeking incubation, there is an increase of CP-AMPAR-mediated synapses in both the core and shell subregions,[31] although the source of these synapses in the core is not clear.[18] Interestingly, while after the incubation of food seeking there is an increase of AMPA/NMDA ratio in the core,[64] a decrease of this ratio is evident in after the incubation of sucrose seeking.[65] We have detected some changes of similar nature but more likely due to the self-administration of the different substances (a reduction of *Gria2*, expression in the core 24 hours after the last session of sucrose self-administration, or increases in the expression of *Gria1*, and the *Gria1*/*Gria2* ratio in the shell after heroin or cocaine self-administration, respectively; see Data S1). Furthermore, the exposure to reward-related cues elicited an enhanced neural response in the core after the incubation of cocaine[66] but not of sucrose[67] seeking.

Since there is no evidence of the involvement of the nucleus accumbens in the incubation of seeking of other drug types (using the same intervention at early and protracted withdrawal), it is possible that this region could only be relevant to the incubation of psychostimulant seeking (as observed by Conrad et al[31] for cocaine and Scheyer et al[68] for methamphetamine), or that these results were consequences of protracted changes in seeking-related pathways not involved in the incubation phenomenon. Therefore, we think that the changes we observed in the nucleus accumbens would not be not related to the incubation phenomenon, instead they would reflect specific effects of the self-administration of the different rewards.

5 | CONCLUSIONS

Not all reinforcers are born equal. Indeed, we show here that, in accordance with the literature, there is little overlap between the neural substrates of the incubation of seeking of psychostimulants, opiates, and a natural reinforcer such as sucrose. Despite this limited overlap, we found that amygdalar alterations to excitatory neurotransmission and endocannabinoid system are common effects of the incubation of seeking that could be exploited to develop new therapeutic approaches to tackle the central problem of relapse in addictive disorders.

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AUTHORS CONTRIBUTION

DRM and AHM designed the experiments. D.R.M., M.U., J.O., I.B.Y., C.A.C., and A.M. performed the behavioral and neurochemical experiments. EA secured the funding for the project and contributed with reagents and equipment. D.R.M. and A.H.M. analyzed the data and wrote the final draft of the manuscript with the help of the rest of the authors.

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