DOCTORAL THESIS

2019

A multiparametric study of the psychobiological mechanisms operating in the incubation of seeking of drugs and natural reinforcers in the laboratory rat

Estudio multiparamétrico de los mecanismos psicobiológicos de la incubación de la búsqueda de drogas y reforzadores naturales en ratas de laboratorio

DAVID ROURA MARTÍNEZ

Doctoral Program in Psychology of Health

Thesis advisors:

Dr. ALEJANDRO HIGUERA MATAS
Dr. EMILIO AMBROSIO FLORES

RESEARCH FUNDED BY:

Spanish Ministry of Economy and Competitiveness:
SAF2013-47520-P
PSI2016-80541-P
PSI2010-20355

Spanish Ministry of Health, Social Services and Equality:

- *Red de Trastornos Adictivos:*
RTA-RD12/028/0020 of the Institute of Health Carlos III

- *Plan Nacional Sobre Drogas:*
2012I057

General Directorate of Research of the Community of Madrid:
S-2011/BMD-2308, Program of I+D+I Activities CANNAB-CM

Universidad Nacional de Educación a Distancia:
Plan for the Promotion of Research
Predoctoral fellowship

European Union:
JUST/2013/DPIP/AG/4823-EU MADNESS

AGRADECIMIENTOS

Estoy profundamente agradecido a mis directores de tesis, Alejandro y Emilio, por la confianza que desde el principio han depositado en mí y por la oportunidad que me dieron de trabajar en su grupo de investigación. Su profesionalidad y calidad humana son encomiables. Así mismo, quiero agradecer a Arturo y María Rosa por confiar en mí y escogerme para investigar con ellos durante el último año y medio de mi estancia en Madrid. Sin lugar a dudas, aquel puesto fue tan oportuno como provechoso.

A continuación citaré, cronológicamente, a aquellas personas con las que he compartido parte de mi trayectoria en forma de compañeros, mentores, amigos y/o enlaces. Cuando cite a varios lo haré en orden alfabético.

La primera persona a quien quiero agradecer es mi madre. Ella me animó a que me mudara, habiendo cumplido la mayoría de edad, de mi pueblo, Pasajes (Gipuzkoa), a Bilbao (Bizkaia), a estudiar aquello que quería, Biología, ya que en el campus de la UPV-EHU de Donostia no lo hay. Gracias a su apoyo, a las becas del Gobierno Vasco (que afortunadamente nunca faltaron) y a trabajar de vez en cuando, pude finalizar los estudios. El segundo cuatrimestre de tercero de carrera cursé Fisiología Animal y recuerdo estar fascinado con la asignatura. Tal es así que cuando vi a la profesora Mertxe colgar un anuncio en el que buscaban candidatos para alumno interno de laboratorio me lancé de cabeza. Y sin fijarme en que era en el departamento de Fisiología, no Fisiología Animal, y en la facultad de Medicina y Odontología, no en la de Ciencias. Seguramente la profesora se limitó a recoger del suelo un anuncio caído. Así que acabé teniendo una entrevista para un grupo del que no tenía ninguna referencia. Y me seleccionaron. A partir de septiembre de 2003 entré como alumno interno bajo la supervisión de María Begoña y José Ignacio, y con ellos obtuve el Diploma de Estudios Avanzados. A ellos les debo mi primer contacto con la ciencia empírica, en un departamento muy bien equipado en el que se cuidaba mucho la transmisión del conocimiento y el trabajo en grupo. Allí tuve la posibilidad de trabajar con muy buenos investigadores, algunos amigos hoy en día: Ainara, Ainhoa, Alejandro', Begoña, Cristian, Fadil, Idoia, Igor, Igotz, Itxaso, Lourdes, Marga, María José, Montse, Nerea, Olatz, Patricia, Rosa, Rosaura, Sandra, Xabier, Yolanda y Yuri (hace tanto que puede que me olvide de alguien).

Pero escaseaba la financiación en mi grupo así que empecé a buscar trabajo en neurociencias, campo que siempre me había atraído, y en Madrid, ciudad que me gustaba y conocía de visita. Fue la mujer de Fadil la que, sin conocerme, encontró una oferta en el laboratorio de Maribel y Esther, en el departamento de Farmacología de la facultad de Medicina de la Complutense. Hice una entrevista, me cogieron y me mudé a la capital. Durante esos tres años (enero 2008-diciembre 2010) tuve la suerte de trabajar con Alejandro (postdoc), David, Elizabeth, Erica (postdoc), Icíar, Nieves y Noelia (postdoc). También a Andrés, amigo de la facultad, que se mudó a Madrid cuando le becaron en el mismo departamento.

Terminándose ya el contrato, Alejandro me comentó que iba a salir una beca conseguida por el grupo de Psicobiología de la Drogadicción de la UNED (en una convocatoria competitiva del Vicerrectorado de Investigación), grupo en el que él se doctoró. Sólo nos presentamos dos personas y, tras casi siete años de experiencia en laboratorio, yo tenía mejor currículum, así que me seleccionaron. Un año después Alejandro se vio codirigiendo mi tesis con Emilio: su primera tesis. Allí también fui agraciado, además de con mis directores, con los compañeros que me encontré, permanentes, duraderos, temporales, estancias, colaboradores, fugaces o amigos: Adrián Emilio, Alberto, Amparo, Ana Belén, Carlos Alberto, Esmeralda, Gonzalo, Gonzalo', Inmaculada, Inmaculada', Javier, Jorge Aldrete, Luis, Luis', Marcos, Mariano, Mario, Miguel, Mónica, Natalia, Nuria, Paula, Raquel, Roberto, Rosa, Santiago, Shishir y Telmo. También pude trabajar, durante una estancia en el instituto Donders (Radboud University, Nijmegen), con Erika, amiga de la carrera que casualmente realizaba la tesis en el grupo de Benno, en el que yo estaba interesado.

Por último, Arturo y María Rosa quisieron contratarme, durante mi último año y medio en Madrid (y en España), para trabajar en el departamento de Toxicología de la facultad de Veterinaria de la Complutense, brindándome la posibilidad de conocer a Arantxa, Bernardo, Irma, José Luis, Natalia, Óscar, Óscar' y Thais. Por casualidades de la vida, pocos meses después de entrar a formar parte del grupo este departamento se fusionó con el de Farmacología de la facultad de Medicina, del que fui miembro años atrás.

Llegar hasta aquí no ha sido fácil, y no sólo ha dependido del esfuerzo que yo haya dedicado a mis estudios y a mi trabajo: también ha sido gracias a la buena voluntad, a la profesionalidad y a la capacidad de gente que por suerte me he ido encontrando por un camino con altibajos en cuanto a financiación.

Por último, agradezco a Dimitris por acompañarme durante todo este difícil proceso, aunque a veces no entendieses muchas cosas del mismo. «Porque los pilares sostienen el templo, pero están separados; y ni el roble ni el ciprés crecen el uno a la sombra del otro».

INDEX

INDEX OF FIGURES, BOXES AND TABLES

1. INTRODUCTION

2. MATERIALS AND METHODS

3. RESULTS

4. DISCUSION

ABBREVIATIONS

2-AG	2-arachidonoylglycerol
4-MTA	4-methylthioamphetamine
5-HIAA	5-hydroxyindolacetic acid
5-HT	5-hydroxytryptamine, serotonin
5HT2C	serotonin receptor 2C
AC	adenylate cyclase
ACC	anterior cingulate cortex
Adra1/a2a/b1	adrenoceptor alpha1/alpha2a/beta1
AEA	anandamide
AI	agranular insular cortex
AIT-L/M	anterior intralaminar nuclei of thalamus, lateral/medial
AIV	agranular insular cortex, ventral
Ala	L-alanine
am	*ante meridiem*
Amg	amygdala
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptor
ANCOVA	analysis of covariance
ANOVA	analysis of variance
ANS	autonomic nervous system
Asp	L-aspartic acid
BA	basal amygdala
β-act	beta-actin
BAU	biochemical analysis in urine
bCBV	basal CBV
BDNF	brain derived neurotrophic factor
BLA	basolateral amygdala
BMP	posterior basomedial amygdala
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumin
Cart, CART	cocaine- and amphetamine-regulated transcript
CB1, *Cnr1*	cannabinoid receptor 1
CBV	cerebral blood volume
cDNA	complementary DNA
CeA	central nucleus of amygdala
CeC	central CeA
CeL	lateral CeA
CeM	medial CeA
CFI	comparative fit index
CI-AMPAR	calcium-impermeable AMPAR
Cnr1, CB1	cannabinoid receptor 1
CPA	conditioned place aversion
CP-AMPAR	calcium-permeable AMPAR
CPP	conditioned place preference
Crh, CRH	corticotropin releasing hormone
CS	conditioned stimulus
CTA	conditioned taste aversion
CTB	Centro de Tecnología Biomédica
CV	covariance
d	day
D1-5, *Drd1-5*	dopamine receptor 1-5
DAGLa, *Dagla*	diacylglycerol lipase alpha
DAPI	4',6-diamidino-2-phenylindole
DAT	dopamine transporter
dBNST	dorsal BNST
dCA1	dorsal cornu Ammonis 1
dCA3	dorsal cornu Ammonis 3
dDG	dorsal dentate gyrus
DEPC	diethylpyrocarbonate
det	determinant
df	degree of freedom
dHPC	dorsal hippocampus
dlPFC	dorsolateral prefrontal cortex
DLS	dorsolateral striatum
DMH	dorsomedial hypothalamic nucleus
dmPFC	dorsomedial prefrontal cortex
DMS	dorsomedial striatum
DNA	deoxyribonucleic acid
Dnmt	DNA methyltransferase
DOR, OPRM1, *Oprm1*	delta opioid receptor
dPFC	dorsal prefrontal cortex
dPL	dorsal prelimbic cortex
Drd1-5, D1-5	dopamine receptor 1-5
DS	dorsal striatum
DSM	Diagnostic and Statistical Manual of Mental Disorders
DTT	dithiothreitol
eCB	endocannabinoid
EDTA	ethylenediaminetetraacetic acid
EE	environmental enrichment
exp	experiment
FAAH, *Faah*	amidohydrolase of fatty acids
FARC	Fuerzas Armadas Revolucionarias de Colombia
FITC	fluorescein isothiocyanate
fMRI	functional magnetic resonance imaging
GABA	gamma-aminobutyric acid
$GABA_A$, *Gabr*	GABA receptor A
Gabra1/a2/g1/d	GABA receptor A
GAPDH, *Gapdh*	glyceraldehyde 3-phosphate dehydrogenase
GDNF, *Gdnf*	glial-derived neurotrophic factor
GHB	gamma-hydroxybutyric acid
Gln	L-glutamine
GLT1	glutamate transporter 1
Glu	L-glutamic acid
GluA1/2, *Gria1/2*	AMPA receptor, subunit 1/2
Gly	glycine
Gria1/2, GluA1/2	AMPA receptor, subunit 1/2
Grin1/2a-b, NR1/2A-B	NMDA receptor, subunit 1/2A-B
Grm1-5, mGluR1-5	metabotropic glutamate receptor 1-5
h	hour
H3K4	histone 3 lysine 4
Hcrt	hypocretin/orexin
Hdac, HDAC	Histone deacetylase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HPA	hypothalamic-pituitary-adrenal
HPC	hippocampus
HPT	hypothalamus
HRP	horseradish peroxidase
Htr, 5HT	serotonin receptor
i.p.	intraperitoneal
I/O curve	input/output curve
IC	insular cortex
IC95	interval of confidence 95%
IL	infralimbic cortex
Ile	L-isoleucine
INN	International Nonproprietary Name
IntA	intermitent access
iRNA	interfering RNA
ISH	*in situ* hybridization
KMO	Kaiser-Meyer-Olkin
KO	knock-out
KOR, OPRK1, *Oprk1*	kappa opioid receptor
LaD	laterodorsal amygdala
LaV	lateroventral amygdala
LC	locus coeruleus
LgA	long access
LH	lateral hypothalamus
LIF	laser induced fluorescence
lOFC	lateral orbitofrontal cortex
LPP	late positive potential
LSD	lysergic acid diethylamide
LTD	long term depression
M	motor cortex
MAGL, *Mgll*	monoacylglycerol lipase
MCH	melanin-concentrating hormone
MePD	posterodorsal medial amygdala
Mgll, MAGL	monoacylglycerol lipase
mGluR1-5, *Grm1-5*	metabotropic glutamate receptor 1-5
MHPG	3-methoxy-4-hydroxyphenylglycol
MLE	maximum likelihood estimation
MOR, OPRM1, *Oprm1*	mu opioid receptor
mPFC	medial prefrontal cortex
mRNA	messenger RNA
MSA	measure for sample adequacy
MSN	medium spiny neuron
NA	noradrenaline
NAc	nucleus accumbens
NAPE-PLD, *Napepld*	N-acylphosphatidylethanolamine phospholipase D
NBD-F	4-fluoro-7-nitrobenzofurazan
NIDA	National Institute on Drug Abuse
NMDA	N-methyl-D-aspartic acid
NMDAR	NMDA receptor
nNOS	neuronal NO synthase
Npy, NPY	neuropeptide Y
NR1/2A/2B, *Grin1/2a/2b*	NMDA receptor, subunit 1/2A/2B
NSF	*N*-ethylmaleimide sensitive fusion protein
NTS	nucleus of the solitary tract
OFC	orbitofrontal cortex
OPRD1, *Oprd1*, DOR	delta opioid receptor
OPRK1, *Oprk1*, KOR	kappa opioid receptor
OPRM1, *Oprm1*, MOR	mu opioid receptor
Orn	L-ornithine
PAGE	polyacrylamide gel electrophoresis
PAM	positive allosteric modulator

PCA	principal component analysis
PDE	phosphodiesterase
Pf	parafascicular nucleus of the thalamus
PF	perifornical hypothalamus
PFC	prefrontal cortex
PKA	protein kinase A
PKC	protein kinase C
PL	prelimbic cortex
PND	postnatal day
PNN	perineuronal net
Pro	L-proline
PSD95	postsynaptic density 95
PV	parvalbumin
PVN	paraventricular nucleus of the hypothalamus
qPCR	quantitative polymerase chain reaction
rCBV	reactive CBV
RIN	RNA integrity number
RMSEA	root mean square error of approximation
RNA	ribonucleic acid
RS	retrosplenial cortex
RT	room temperature
s.c.	subcutaneous
SA	self-administration
SCH	saline cocaine heroin
SD	standard deviation
SDS	sodium dodecylsulfate
SE	standard error
SEM	structural equation modelling
ser	serine
ShA	short access
shRNA	short hairpin RNA
siRNA	small interference RNA
Sk2	small conductance calcium-activated K channel 2
SMC	squared multiple correlation
SNpc	substantia nigra pars compacta
SNpr	substantia nigra pars reticulata
SRW	standardized regression weight
SS	somatosensory cortex
Tau	taurine
TCE	trichloroethanol
TEMED	*N*,*N*,*N*',*N*'-tetramethylethane-1,2-diamine
Th	thalamus
TH	tyrosine hydroxylase
THC	tetrahydrocannabinol
Thr	L-threonine
TLR4	Toll-like receptor 4
TrkB	tropomyosin receptor kinase B
tRNA	transfer RNA
UNODC	United Nations Office on Drugs and Crime
US	unconditioned stimulus
UV	ultraviolet
v	volume
vBNST	ventral BNST
vHPC	ventral hippocampus
vmPFC	ventromedial prefrontal cortex
vOFC	ventral orbitofrontal cortex
vPL	ventral prelimbic cortex
vSub	ventral subiculum
VTA	ventral tegmental area
w	weight
WB	western blot
wd	withdrawal
WT	wild type
WZ	water sucrose

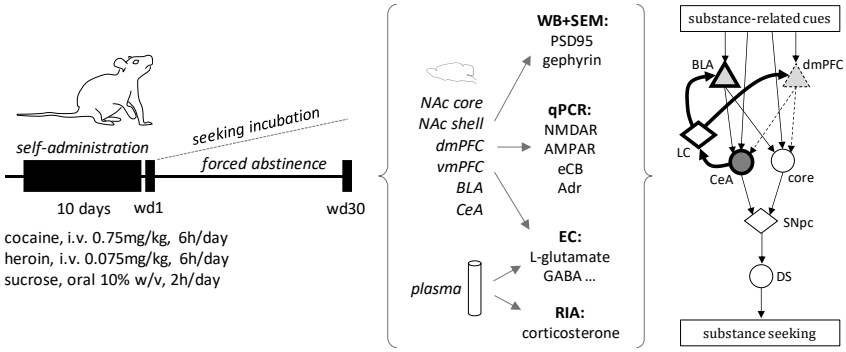
ABSTRACT

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 modelled 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 male rats were used to study the effects of cocaine, heroin and sucrose seeking incubation on 6 key brain regions: the nucleus accumbens shell and core, the central nucleus of amygdala and basolateral amygdala, and dorsomedial and ventromedial prefrontal cortex. There were three main goals: first, to find behavioural parameters during the self-administration sessions that correlated with the strength of incubation; second, to look for common changes in parameters related to regional activity; third, to study stress-related parameters in these areas.

For the first objective, we extracted behavioural parameters and calculated new ones from the self-administration data, but no one correlated with the incubation degree. For the second objective, we analysed PSD95 and gephyrin scaffolding protein levels and the relationships between the areas were examined by Structural Equation Modelling. Also, gene expression of glutamatergic, GABAergic and endocannabinoid elements were analysed as well as amino acid transmitters levels and prefrontal perineuronal net densities. For the third objective, we analysed peripheral parameters (body weight, hepatic and splenic indices, adrenal gland size and plasmatic corticosterone) as well as the gene expression of adrenoceptors in the brain areas.

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. Furthermore, we found alterations in the central nucleus of amygdala and the glutamatergic regions mentioned above related with their activity under high arousal states. These results suggest a key role for the central nucleus of amygdala and its cortical and amygdalar afferences in the incubation phenomenon and for their endocannabinoid and noradrenergic systems.



1. INTRODUCTION

1.1. THE PROBLEM OF DRUGS OF ABUSE AND RELAPSE

"*The critical point of withdrawal is not the early phase of acute sickness, but the final step free from the medium of junk… There is a nightmare interlude of cellular panic, life suspended between two ways of being… At this point the longing for junk concentrates in a last, all-out yen, and seems to gain a dream power: circumstances put junk in your way… You meet an old-time Schmecker, a larcenous hospital attendant, a writing croaker…*" (William S. Burroughs, *Naked Lunch*, 1959)

1.1.1. The problem of relapse

Addiction is considered a chronic psychopathology due to its high relapse rate. In recent years, only in Europe, around 25 000 people have started cocaine detoxification treatment every year, and it rises to 30 000 in the case of heroin. The vast majority of them are 34 year-old men. For more than half of them it was not the first time (Figure 1).

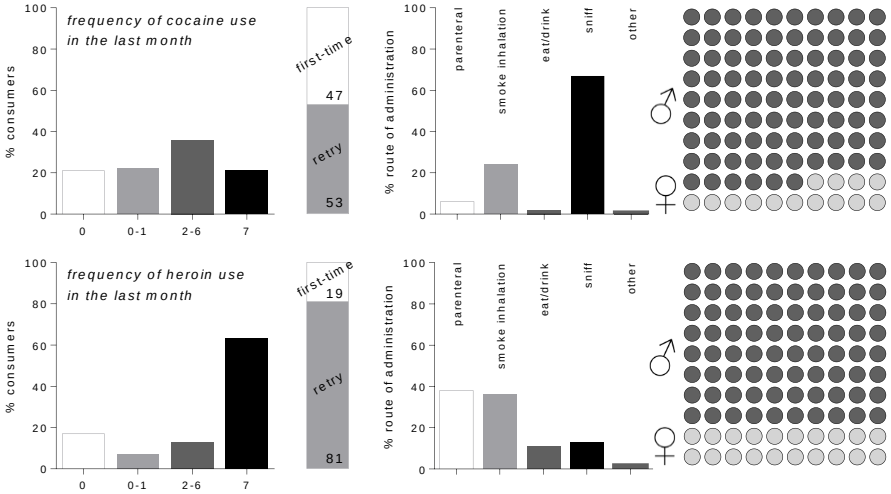


Figure 1. Profile of people who initiate treatment in Europe. *From left to right*: frequency of weekly consumption (0-7 days per week); distribution of patients according to whether it is their first treatment (first-time) or not (retry); routes of administration of the drug; distribution of sexes (each circle equals 1%). *Above*: cocaine; *down*: heroin Approximate total equals to: cocaine, 25 000 people; heroin, 30 000 people. Source: European report on drugs 2017 (2015 data).

When contemplating a survival curve in which the length of abstinence is represented in the axis of ordinates and the percentage of abstainers in the abscissa axis, we can observe how certain patterns are followed independently of the substance to which people are addicted. After six months half of the individuals have already relapsed, and during the following year of abstinence the number rises steadily. The shape of the curves varies little between substances and have remained similar over decades (Brecht and Herbeck, 2014; Hunt *et al.*, 1971; Sinha, 2011). In less than two years four out of five individuals have suffered a relapse (Figure 2).

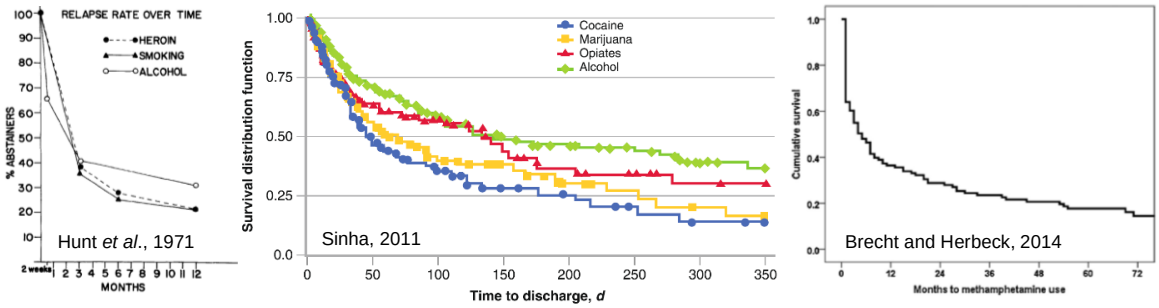
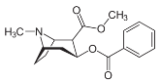


Figure 2. Survival curves. From at least the study of Hunt *et al.* (1971) in which they studied the relapse in addicts to heroin, tobacco or alcohol, the curves have followed the same pattern.

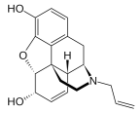
This shows us that therapies against addiction have hardly improved over time. In the end, the individual is immersed, in most cases, in a cycle of abstinence-relapse-treatment, which reflects the chronicity of this psychopathology. Since relapse is a central milestone in drug addiction, understanding its causes and dynamics throughout withdrawal is crucial for therapeutic intervention.

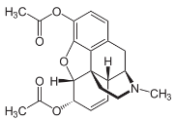
Box 1. Coca and cocaine



Cocaine (benzoylmethylecgonine, INN) is a tropane alkaloid (such as belladonna atropine or scopolamine and mandrake hiosciamine) found in the leaves of coca (Quechua *kuka*, *Erythroxylum coca*) that synthesizes it from of the amino acid L-phenylalanine. This plant is native to the Amazonian Andes, current Colombia, Ecuador, Peru and Bolivia. Like opium, coca has been used by peoples before their main active compounds were isolated. The most common use of the leaves is to chew them, which prevents altitude sickness. It is also used as an anaesthetic. Its production is persecuted because of it cocaine is extracted, with whose contraband terrorist groups like the FARC are financed. The first to isolate and identify this alkaloid was Albert Niemann in 1859. It began to be used to treat addiction to morphine in 1879 (also because it was believed to be less addictive) and as an anaesthetic by 1884. The desired effects of cocaine are happiness and extreme energy, state of mental alertness and hypersensitivity of sight, hearing and touch. In the mid-twentieth century cocaine addicts were already hundreds of thousands and today it is the second most consumed illegal drug in the world behind cannabis (although far behind the legal ones: alcohol, tobacco and hypnosedatives). Acute side effects are irritability, paranoia, restricted blood vessels, dilated pupils, nausea, elevation of body temperature, blood pressure and heart rate, tremors, muscle contractions and restlessness. The long-term effects are malnutrition (since it decreases appetite), Parkinson's type movement disorders (after many years of use), irritability, restlessness, paranoia and those derived from the route of administration: loss of smell, nosebleed , problems with ingestion, deterioration of the intestines, or HIV and hepatitis C (NIDA).

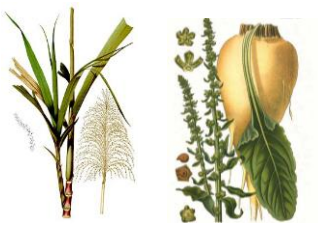
Box 2. Opium, morphine and heroin





Heroin (diacetylmorphine, INN) is synthesized in the laboratory from the acetylation of morphine hydrochloride, an alkaloid present in opium. This is a resin that is extracted from incisions made in the green capsules without petals of the opium poppy (*Papaver somniferum*), of the same genre as the common poppy (*P. rhoeas*). Morphine is a benzylisoquinoline alkaloid (such as papaverine, thebaine, codeine or noscapine, all present in the opium poppy and therefore called opiates) that the plant synthesizes from the amino acid L-tyrosine. The opium poppy is native to the Mediterranean basin and Fertile Crescent. There is evidence that opium has been used for millennia by different cultures. One of its effects, shared with THC, the active ingredient of hemp (*Cannabis sativa*), is the analgesic, as well as pleasant sensation. The opium trade led to serious conflicts such as the Opium Wars, in which the British Empire, the United States, and France fought against China, which suffered an epidemic of opium addiction and tried unsuccessfully to stop its contraband. The first to isolate and identify morphine (*Morpheus*, Greek god of sleep and dreams) was the German Friedrich WA Sertürner, who marketed it from 1817. After the invention of the hypodermic syringe in 1853 began to be used as an analgesic, especially in the war, it left hundreds of thousands of morphine addicts (soldier's disease). In 1878 Charles RA Wright synthesized and isolated heroin, which began to be marketed by Bayer in 1898 as a cough suppressant and to treat morphine addiction. Shortly after the first cases of heroin addicts began to be known, experiencing a worldwide boom in the decades of the 1970s and 1980s, which caused the death of thousands of young people especially in the West. As of the 1990s, heroin abuse fell due, among other factors, to the appearance of AIDS and the increasing use of cocaine. Although its use is marginal today it has grown during the Great Recession (2008-present) and in the USA there is talk of an epidemic. Acute side effects are slowing of heart rate and breathing, dry mouth, redness and overheating of the skin, feeling of heaviness in arms and legs, nausea and vomiting, intense itching, clouding of the mental faculties and repeated alternation between waking states and numbness. Long-term side effects are insomnia, infection of the pericardium or heart valves, abscesses, constipation, stomach pain, liver and kidney diseases, pulmonary complications such as pneumonia, mental disorders such as depression or antisocial personality disorder, sexual dysfunction in men and irregular menstrual cycles in women, in addition to those derived from the route of administration: HIV and hepatitis C, collapse of veins in which it is injected, or damage to the tissues of the nose (NIDA).

Table 3. Sugar and sucrose



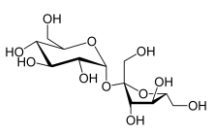


Table sugar or sucrose (α-D-glucopyranosyl-(1→2)-β-D-fructofuranoside) is the molecule used by plants to move carbohydrate reserves between the different organs through the sap, of the same way animals use glucose through the blood. Animals do not synthesize it but are able to process it. Sugarcane (*Saccharum officinarum*), native to Southeast Asia, and sugar beet (*Beta vulgaris* ssp, *vulgaris* var. *altissima*), native to the European continent, are plants cultivated to produce it in large quantities. The first to extract and purify it were from India. From there the technology travelled to Persia and from there, with the Muslim expansion, to Europe. Then it crossed the ocean to America, the world's leading producer. The sugar beet began to be used from the eighteenth-nineteenth centuries in order for metropolis to be independent from their colonies (IEDAR, Institute of Studies of Sugar and Beet). Another traditional sweetener is honey, which, being of animal origin, contains mostly fructose and glucose. At the end of the 19th century, synthetic and semi-synthetic sweeteners began to appear (saccharin: 1879). These molecules bind to membrane receptors on the tongue, palate and epiglottis, as do natural sweeteners. But unlike them they contain few (or none) calories. Increasingly, both natural and synthetic sweeteners are added to processed foods for later consumption. At the same time, more and more studies are published in which one or the other is associated with health problems such as diabetes, cavities, different metabolic disorders or even cancer. The creation of a sugar tax has been raised ("Obesity: We need to move beyond sugar," 2016).

1.1.2. Effects of timing on relapse

"*Con la sola excepción de los alimentos, no existen en la Tierra sustancias que estén tan íntimamente asociadas a la vida de los pueblos en todos los países y en todos los tiempos*" (Ludwig Lewin Jacobson in Jean Louis Brau's *Historia de las drogas*).

Substances of abuse have existed for millennia and have been consumed throughout history for many reasons (Boxes 1-4). They are anchored to the culture of peoples in the form of ointments, potions or catalysts that accompany rites and other social interactions, such as drinking alcohol, smoking tobacco when leaving bars or consuming ayahuasca in certain rituals. Also in the form of (self)medications such as chewing coca leaves (*chacchar*) to prevent altitude sickness or to consume opium or marijuana to fight pain.

When an individual with a problematic substance use ceases to consume, it is said that she/he enters a period of abstinence. This period can be interrupted momentarily, what we call *slip* or *lapse*, or consumption behaviour can be restored, which is called *relapse*. More often, lapse follows a relapse. This lapse and subsequent relapse can be triggered by the reasons mentioned above (ritual, medication). If the interruption of the consumption causes a syndrome of abstinence, the relapse can be a way to avoid it. To these factors is added exposure to stimuli and contexts strongly associated with drugs (an ashtray, a bar) and stress situations (Dong *et al*., 2017). It has been observed, for example, that periods of economic recession are associated with an increase in drug use and that this association is due to the distress caused by the loss of employment or the fear of losing it (Nagelhout *et al*., 2017). Drug abuse carries a series of negative consequences (Boxes 5-6). Therefore, the understanding of the causes of relapse is crucial to combating them.

The intensity of craving may vary at the individual level depending on various timing factors, such as the time of day, the frequency of use and the duration of abstinence.

Circadian effects on heroin craving. In their study, Ren *et al*. (2009) measured the ability of heroin associated cues to induce craving throughout the day, from 8:00 am to 8:00 pm They recruited 80 heroin addicts, all men, aged between 20 and 45 years old, retained in a rehabilitation centre in Beijing. They observed a decrease in craving towards midday, which correlated with induced anxiety. None of these parameters correlated with changes in heart rate or diastolic or systolic pressures after being exposed to these cues.

Effect of the frequency of cocaine and alcohol consumption on craving. In their study, conducted at the Connecticut Mental Health Centre, Fox *et al*. (2005) divided individuals (54 cocaine addicts of both sexes) into high and low frequency consumers. They observed that high frequency consumers, who also consumed more alcohol, were more sensitive to stress and cues to craving and anxiety. A few years earlier, Ahmed *et al*. reported that rats that had escalated their use during cocaine (S H Ahmed and Koob, 1999) and heroin (Ahmed *et al*., 2000) self-administration protocols with extended access (6 hours a day) were more sensitive to reacquire consumption behaviour after an episode of stress than rats that had acquired the habit with restricted access protocols (1 or 2 hours a day).

Box 4. Origin of drugs of abuse

Where do the so-called drugs of abuse come from? There are natural, synthetic and semi-synthetic (from natural substances). Human beings have consumed them for millennia. The origin of many of the natural substances of abuse is closely linked to food: it is thought that to prevent being devoured by herbivorous animals, nature began to select the plants based on their ability to repel them. Although attractant molecules appeared to promote zoochory and thus take advantage of their predators, a plethora of repellent, toxic, psychotropic secondary metabolites also emerged, which the animals had to face. Toxics as the capsaicin, as well as neurotoxics acting on different neurotransmitter systems. Among them are morphine (from which heroin is synthesized), thebaine (from which buprenorphine is synthesized), cocaine, nicotine, caffeine, ephedrine (from which amphetamines are derived), cannabinoids, ergoline (from which LSD is obtained) and a long etcetera. But why did reinforcement mechanisms evolve in mammals that are easily triggered by toxic substances? Some researchers explain this paradox of drug reward appealing to a counter-exploitation of plants neurotoxins by animals to fight parasites (*pharmacophagy*), as analgesics or as cognitive enhancers; "*Conversely, unexploited toxins will not activate reward and reinforcement mechanisms*" (Hagen *et al*., 2009).

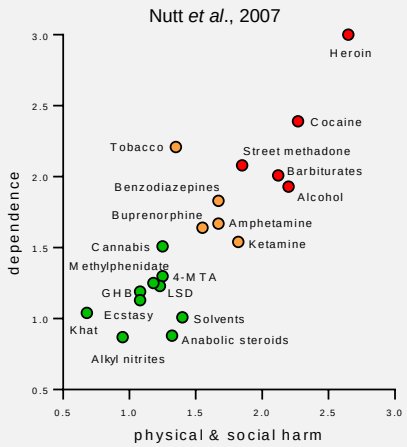
But not all drugs of abuse with a natural origin come from the secondary metabolism of the plants: both alcohol and GHB come from fermentations, and are also synthesized to a lesser extent in animals. Among synthetic substances of abuse we can mention barbiturates, benzodiazepines, methadone (opioid), ketamine and inhalants (organic solvents, alkyl nitrites), although there are also of geological origin, such as the ethylene of the Oracle of Delphi (De Boer and Hale, 2000). In addition to the substances of abuse mentioned above, food in general, and sugar in particular, can also generate compulsive behaviours.

Effect of the abstinence length on craving. There are five publications (three from China and two from the United States, Table 1, Figure 3) about the evolution of the basal craving and the cue-induced craving throughout the abstinence period of people addicted to cocaine (Parvaz *et al*., 2016), methamphetamine (Wang *et al*., 2013), heroin (Wang *et al*., 2012), alcohol (Li *et al*., 2015c) and tobacco (Bedi *et al*., 2011). Other parameters studied can be grouped into two main blocks: the withdrawal syndrome and related parameters, such as cortisol levels, heart rate, emotional state, anxiety and depression; and neuropsychological measures related to decision making, attention, impulsivity and work memory.

Reference	Drug of abuse	Type of study	Control of abstinence	Regime	Measurement of craving
Parvaz *et al*., 2016	cocaine	transversal	questionary	none	questionary (liking, wanting),LPP
Wang *et al*., 2013	metamphetamine	transversal	questionary	prison	questionary
Wang *et al*., 2012	heroin	transversal	questionary	prison	questionary
Bedi *et al*., 2011	tobacco	longitudinal	questionary + BAU	none	questionary
Li *et al*., 2015c	alcohol	longitudinal	questionary + BAU	hospital	questionary

Table 1. Studies about human craving incubation. BAU: biochemical analysis in urine; LPP: late positive potential.

In general, it is observed that the basal craving decreases as the abstinence progresses, in parallel to the withdrawal syndrome and the errors in decision making. On the contrary, cue-induced craving increases during abstinence, to go back down after a few months, a phenomenon called *incubation of craving*.



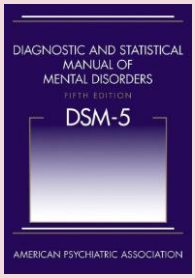
Box 5. Problems associated with drug use

It is estimated that a total of 246 million people (or one in 20 people between the ages of 15 and 64) consumed illicit drugs in 2013. As indicated in the World Drug Report (UNODC, 2017), the magnitude of the problem becomes more evident if one takes into account that more than 1 in 10 drug users is a problematic consumer suffering from disorders caused by drug use or drug dependence. That is, around 27 million people are problematic drug users worldwide. Practically half of them use injectable drugs, and an estimated 1.65 million of them were affected by HIV in 2013. To this virus must be added that of hepatitis C. The annual number of deaths related to drug use (187 100 in 2013) has hardly changed. Undoubtedly death by overdose is the worst consequence of substance use. Although in 2010 the numbers of overdose deaths in Europe began to fall (from 8000 to less than 7000), there has been an upturn, exceeding the 8000 deaths in 2015. Four out of every five deceased are men, and the vast majority with the presence of opioids. To this must be added the financing of organized crime groups derived from the consumption of illicit drugs, especially heroin and cocaine. And although there is no direct relationship between risks and legislation, these two, heroin and cocaine, are without a doubt the most harmful in terms of dependence and physical deterioration (Nutt *et al*., 2007).

Box 6. DSM-5: Substance use disorder and compulsive eating disorder

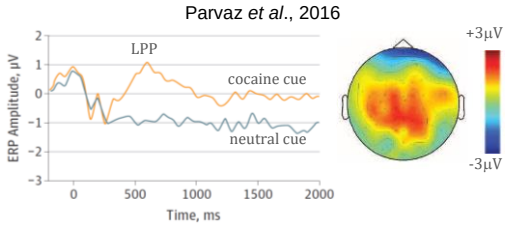
According to the US National Institute on Drug Abuse (NIDA), "*drug addiction is a chronic disease characterized by drug seeking and use that is compulsive, or difficult to control, despite harmful consequences*." In the DSM-5 (American Psychiatric Association, 2013) we can find substance-related and addictive disorders' and 'feeding and eating disorders'. They have in common a phase of compulsive consumption followed by abstinence and relapse due to craving, which can be induced by stress.

Substance use disorders. In the latest version of the DSM the notion of degrees of substance used disorder is introduced, depending on the number of diagnostic criteria that the individual meets (low, moderate, severe). There are criteria related to abuse (appearance of social problems, risky use, health problems), dependence (tolerance, withdrawal syndrome, escalation) and craving. Among these criteria, we find the 'repeated attempts to stop or control consumption', alluding to the cycles of abstinence and relapse that drug addicts present.



Compulsive eating disorder Also called binge-eating disorder, it is undoubtedly the most similar to the previous one. The essential characteristic is the presence of recurrent episodes of unnecessary binge eating with loss of control. In U.S.A. it has a prevalence of 1.6% in women and 0.8% in men, without differences between cultural groups or with other industrialized countries. It is often suffered from childhood or adolescence and there seems to be some genetic predisposition. It is often accompanied by obesity, which is already considered a pandemic and a form of severe malnutrition.

1.1.3. Clinical relevance of the incubation of craving



Research in human craving incubation. In the previously mentioned study with cocaine addicts (Parvaz *et al*., 2016), three parameters related to craving were measured: the so-called liking and wanting, and the cue-related late positive potential (LPP). While the first two followed the same kinetics as the basal craving, the last, related to craving and relapse, suffered an increase during the first months to fall after a year.

In the study of heroin addicts (Wang *et al*., 2012), no such phenomenon was observed, possibly because the first time they measured the induction of craving was one month. For that time it is possible that the phenomenon had reached a maximum (Gu, 2018). On the other hand, this study is transversal, not longitudinal, and in addition no biochemical tests were performed to verify the abstinence of the participants. There is at least one other study in heroin addicts in which an incubation of craving is observed, although they do not distinguish between basal and induced craving (Nava *et al*., 2006). In this study they observed a decrease in craving from the first day to the third month followed by an increase after one year of abstinence. In another study on cigarette smokers they also observed an initial decrease and a subsequent increase in the second month of abstinence from the severity of the withdrawal syndrome in half of the participants (Piasecki *et al*., 2000).

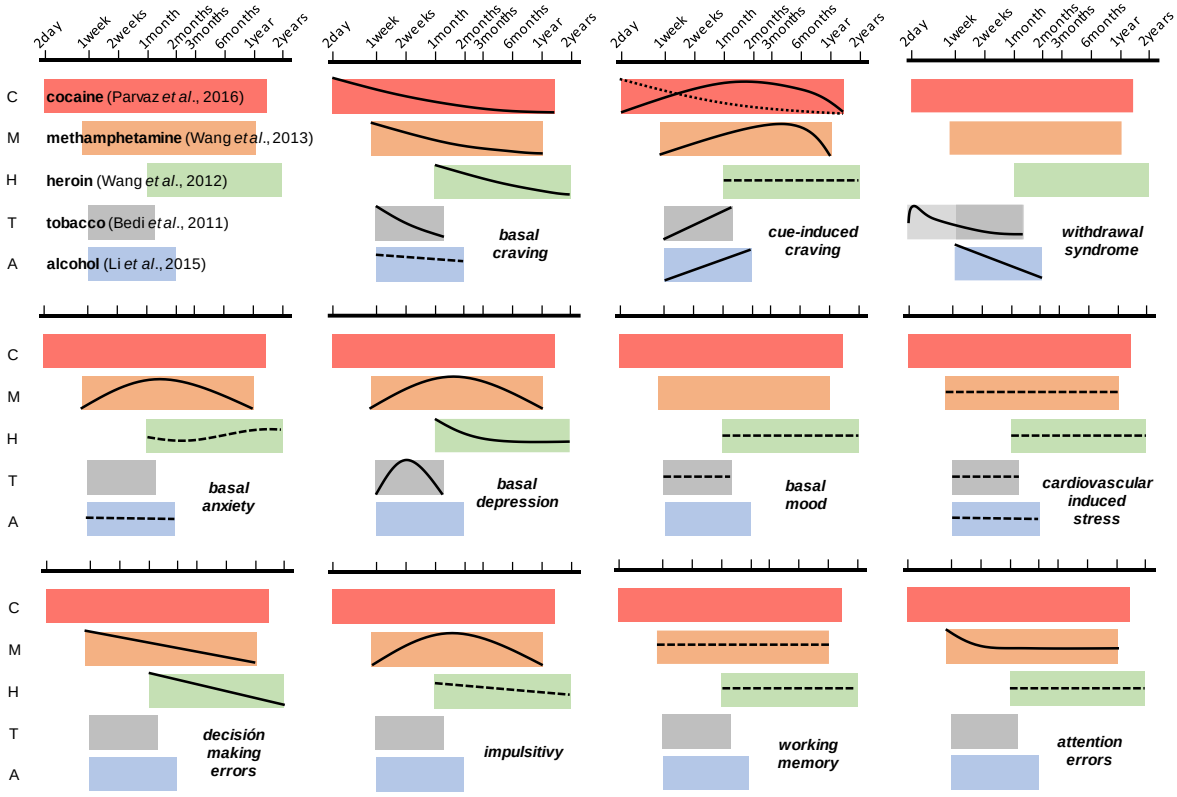


Figure 3. Parameters measured in studies on incubation of craving in addicted people. Evolution of different parameters is presented over time for the five drugs of abuse (C: cocaine; M: methamphetamine; H: heroin; T: tobacco; A: alcohol). Solid lines, changes with significant differences; dashed lines, without significant differences; dotted line in the craving induced by cues associated with cocaine represents the value of liking and wanting.

As early as 1986, researchers Gawin FH and Kleber HD hypothesized that induced craving could present this incubation property (Li *et al*., 2016). However, it was not until the decade of 2010s, ten years after its discovery in animals, that the study of the incubation of craving in humans began.

Clinical relevance in the relapse of drug abuse. The evolution of induced craving throughout the abstinence period could have important therapeutic implications to avoid relapse. Accordingly, over the last decade more than 100 articles on the subject have been published. The actual shape of the incubation curve for drugs of abuse is unknown because the studies available on this matter are longitudinal and of short duration (tobacco and alcohol) or long-term cross-sectional (cocaine and methamphetamine). In this type of studies there is an obvious bias: participants with long abstinence times are those who are more resistant to relapse, and therefore a lower score is expected in the measure of craving. In addition, in none of them was abstinence verified with biochemical tests. Therefore, it is possible that the incubation of craving could have been maintained for at least one year of abstinence.

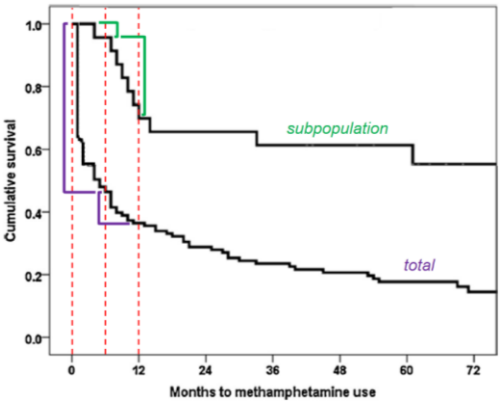


Figure 4. Curve of survival/abstinence for methamphetamine abstinence. For the total population the greatest fall occurs during the first six month, and during the next six month for the subpopulation with less vulnerability (Brecht and Herbeck, 2014).

Does this mean, therefore, that the probability of relapse will increase with the time of abstinence? Referring again to the survival/abstinence curves we observed that this is not the case. This is somewhat expected as the basal craving is at its maximum at short times of abstinence and then decreases. In addition, there are other triggers of relapse such as withdrawal syndrome and stress.

In the study on methamphetamine addicts, Brecht and Herbeck (2014) observed that, although the survival/abstinence curve of the total individuals responded to the pattern described above, when circumscribing the study to those individuals with family environments of low vulnerability to relapse and with psychotherapy during abstinence the greater probability of relapse did not occur in the first six months after stopping consumption but in the following six months of abstinence (Figure 4). However, there are no more studies that are limited to this subpopulation with this or another drug, so the relationship with the incubation of craving is mere speculation.

Clinical relevance in the relapse of food consumption. The incubation of food craving induced by food-related cues is elusive mainly because it has never been studied as such and, therefore, the experimental designs are not optimized to detect it. An added problem is to define food abstinence, given the inevitability of eating in daily life. A valid approach could be to study people on diets: individuals subjected to restrictive ones are more abstainers than people subjected to normal, non-restrictive diets. By studying groups with restrictive diets, it has been observed that basal craving, like that experienced with drugs of abuse, tends to decrease during abstinence. At least three studies observe this effect, measuring the craving at different times each: at 0, 6 and 12 weeks (Martin *et al*., 2006), at 1 and 7 days (Massey and Hill, 2012), and at 0 and 6 months (Batra *et al*., 2013). A clue about the existence of the incubation of food craving induced by cues is found in the metanalysis conducted by Boswell and Kober (2016). When calculating the craving induced by food-related cues in people without and with restrictive diets, they found a higher although non-significant value (p=.09) in the seconds (unrestricted: r=.19, IC95=[.10,.28]; restricted: r=.31, IC95=[.20,.42]; Figure 5).

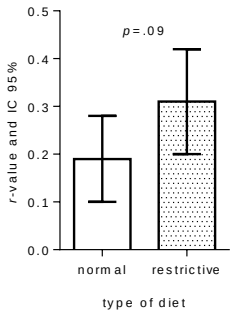


Figure 5. Craving during diet. Cue-induced food craving appears to be higher in people undergoing restrictive diets than during normal diets (Boswell and Kober, 2016).

There is an equivalent phenomenon: the *incubation of the fear response*. This phenomenon is known at least since the beginning of the 20th century (Diven, 1937) and is qualified as necessary for the symptoms of a trauma to be expressed. If both incubations, that of conditioned fear and that of cue-induced craving, shared underlying processes and these were of equivalent intensity, we could say that the incubation of craving is a relevant phenomenon in relapse. Again, in the absence of studies that directly contemplate this possibility, it is mere speculation.

1.2. STATE OF THE ART IN THE INCUBATION OF CRAVING

1.2.1. Human neuroimaging studies

Whether we talk about food or about drugs of abuse such as tobacco, the cues associated with the substance induce desire to consume, and ultimately, consumption. Such is the case of the association between the reactivity to these stimuli, measured by functional magnetic resonance imaging (fMRI), and the relapse to tobacco consumption (or persistence to smoking) in smokers. In the case of food, there is a relationship between cue-reactivity, weight and the risk for obesity. In fact, it has been observed that the stimuli related to smoking and food activate the same brain regions: the amygdala, the hippocampus, the prefrontal cortex and the striatum. The greatest overlap is found in the amygdala and the ventral striatum, although it seems that the activation of the latter by the stimuli declines with abstinence (Tang *et al*., 2012; Figure 6). In a review about the relationship between reactivity to associated stimuli and relapse and treatments, they found certain similarities between the different drugs of abuse. First, relapse seems to be related to a greater cue-reactivity of the dorsal prefrontal cortex (dPFC). Second, although with less evidence, pharmacological treatments seem to cause decreases in the reactivity of the ventral striatum and the orbitofrontal cortex. Finally, psychosocial treatments seem to decrease the reactivity of both the dPFC and the amygdala (Courtney *et al*., 2016). However, given that the incubation of cue-induced craving is a process that develops throughout the time of abstinence, it is important to determine how the reactivity of the brain regions changes with the associated stimuli. We have only found two articles in which the reactivity to the cues after short and long periods of abstinence is analysed (including the explicit differences between both timepoints). The first of these two studies deals with heroin addicts and the second with people on diets.

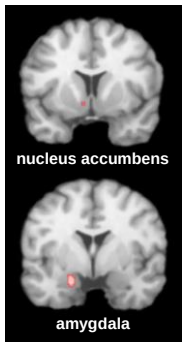


Figure 6. Brain regions activated in fMRI studies. Both the stimuli associated with smoking and those associated with food activate the amygdala and nucleus accumbens (Tang *et al*., 2012).

Studies of fMRI during heroin abstinence. In a study in which the reactivity to heroin-related cues was measured by fMRI in short-term (7-72 days) and long-term (150-300 days) heroin abstinence patients, it was observed that the latter had a lower activation of the caudate and the cerebellum, as well as different cortical regions (anterior cingulate cortex, inferior parietal lobe, medial prefrontal cortex, medial occipital, precuneus). Furthermore, only in the group of long-term abstainers a greater reactivity of the amygdala was observed (Li *et al*., 2013a).

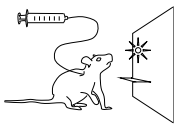
Studies of fMRI during food dieting. In a recent study, Kahathuduwa *et al*. (2018) analysed, not reactivity, but functional connectivity between regions after exposing subjects with or without restrictive diets to food-related cues. Specifically, they observed that the control exercised by the dorsolateral prefrontal cortex (dlPFC) over other areas, such as the orbitofrontal cortex, the amygdala and the nucleus accumbens, during exposure to visual cues related to food was strongly correlated with less craving during a diet of total food replacement when compared to a typical low calorie diet that fails to reduce craving to the same levels as the first. In the rat, the medial prefrontal cortex (mPFC) presents neurophysiological similarities with primate dlPFC (Seamans *et al*., 2008), so the weaker control of mPFC over these areas could be related to a greater craving induced by cues.

1.2.2. Animal models of craving incubation

"*A real-life heroin market differs from the controlled supply environment in animal experiments and is a product of social behavior*." (Hoffer *et al*., p.318, *Computational neuroscience in drug addiction*)

Animal models of consumption, abstinence and relapse. There are several paradigms, differing mainly in whether the animal receives the dose actively or passively:

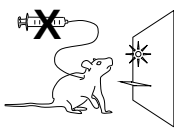
i) Passive administration (non-contingent). The animal receives a dose when the researcher wishes, independently of the behavior of the animal. Because drug administration does not depend neither on the action nor the motivation of the animal, this model is usually employed to study pharmacokinetic and pharmacodynamic aspects of drugs, such as tolerance and sensitization. The place conditioning, both preferential and aversive (CPP, CPA), is a test that is performed in a device composed of two compartments joined by a corridor. The animal receives a non-contingent dose of a substance in one of the chambers and then the researcher observes the animal's preference for staying on one or the other, interpreted as preference or aversion for the substance. With this paradigm the Pavlovian component of addiction can be studied, and although the rat is also used, the mouse is traditionally the experimental subject in this paradigm. Another variant of passive administration is the study of locomotor sensitization, that is, the increase in locomotor activity after several administrations of the drug.



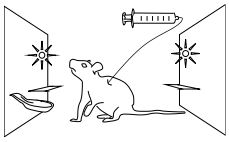
ii) Self-administration (contingent). The animal receives a dose each time it performs a certain action (such as pressing a lever or inserting the head into a cavity, usually associated with a stimulus like a light or tone) in a Skinner box. Therefore, with this paradigm instrumental components of addiction are studied, and although the mouse is also used, the rat is traditionally the experimental subject in this paradigm. Different psychological phenomena such as decision making, lack of control, compulsivity and habituation can be studied. The duration of the daily sessions affects the addictive phenotype obtained in these rodent models. Indeed, the phenotype most similar to the human situation is the one obtained with longer session times (≥4 hours a day, extended access) rather than with short times (≤2 hours a day, restricted access). For example, after a first phase of behavior acquisition (for example, pressing a lever to receive an intravenous drug dose), comes a second phase of behavior maintenance in the case of restricted access, and escalation in the behavior in the case of extended access, reproducing the situation in humans. Also after extended access, and as in humans, rats will show a stronger preference for the drug than for other natural reinforcers (eg, sucrose), and will be more resistant to a punishment afflicted by consumption, that is, they will keep more time consuming despite receiving a punishment for doing so. This last sign can be used as a criterion when diagnosing rodents as addicts. In addition, as we will see in the next section, rats that go through an extended access protocol are more likely to incubate cue-induced seeking.

Both the paradigms of passive administration and self-administration can be combined with periods of abstinence:

iii) Withdrawal. The animal is returned to its home cage, separated from the context and the cues associated with the administration.

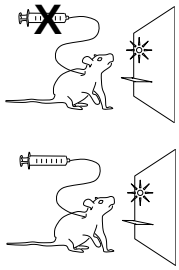


iv) Extinction (only in cases of CPP and self-administration). The animal is exposed to the context and/or the cues associated with the substance but in the absence of it. Throughout the sessions the animal will cease to hang around that chamber or press that lever. The clinical relevance of this last paradigm is doubtful, given that drug addicts do not usually experience extinction. Therefore, and although most published articles on relapse use extinction protocols prior to relapse, withdrawal is increasingly used.



v) Voluntary abstinence. The animal stops self-administering the substance because it is given a choice between it and preferring palatable food (Caprioli *et al*., 2015a).

After a period of abstinence, both relapse and reacquisition can be studied. Both can be triggered by a passive administration of the drug, by stress, by exposure to the context or by exposure to the cues associated with consumption:



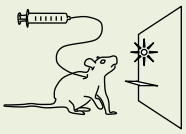
vi) Relapse. Although the term may be regarded as misleading, the term seeking is commonly used for protocols in which the animal is placed into the Skinner box but no drug is available. The active lever and stimuli may be present when pressed, or only the lever. Therefore, this situation is actually an extinction session.

vii) Re-acquisition. When this term is used it is to refer to relapse protocols in which the drug is present contingently.

Animal models of seeking incubation. The first investigations that observed the incubation of seeking in rodents were carried out in rats (*Rattus norvegicus*) with intravenous self-administration protocols with extended access to the substance, cue-induced relapse and no extinction during abstinence. Since then, this phenomenon has been observed in other paradigms. Below are the representative studies of each of them (carried out in rats unless otherwise indicated) and the substances with which they were observed, as well as some cases in which they were not observed (Box 7).

Box 7. Paradigms in which incubation has been observed

a. ***SELF-ADMINISTRATION…*** (contingent)



a.1. <u>*Extended access*</u>…

a.1.1. *and withdrawal followed by*…

a.1.1.1. *relapse*…

… induced by cues:

- cocaine (Grimm *et al.*, 2001)
- heroin and sucrose (Shalev *et al*., 2001)
- sucrose (Grimm *et al.*, 2002)
- methamphetamine (Shepard *et al*., 2004)
- alcohol (Bienkowski *et al*., 2004)
- nicotine (Abdolahi *et al*., 2010)
- sucrose (Aoyama *et al*., 2014)
- palatable food (Krasnova *et al*., 2014)
- WIN55,212-2 (Kirschmann *et al*., 2017)
- oxycodone (Blackwood *et al*., 2018)

… induced by context:

- heroin (Sun *et al*., 2015)
- *not with* methamphetamine (Adhikary *et al*., 2017)

… induced by stress:

- heroin (Shalev *et al*., 2001)
- *trend with* sucrose (Shalev *et al*., 2001)
- *not with* methamphetamine (Shepard *et al*., 2004)

… induced by a non-contingent dose:

- *not with* methamphetamine (Adhikary *et al*., 2017)

a.1.1.2. *readquisition:*

- alcohol (Bienkowski *et al.*, 2004)
- heroin (Zhou *et al*., 2009)
- palatable food (McCue *et al.*, 2018)
- *cocaine (Guillem and Ahmed, 2018)
- **not with* cocaine (Hollander and Carelli, 2005)

a.1.2. *and voluntary abstinence followed by cue-induced relapse*:

- methamphetamine (Caprioli *et al*., 2015a)
- *not with* heroin (Venniro *et al.*, 2017b)

a.1.3. *and extinction followed by cue-induced relapse:*

- cocaine (Madsen *et al*., 2017)
- nicotine (Markou *et al.*, 2018)

a.1.4. *and context extinction followed by cue-induced relapse:*

- sucrose (Harkness *et al*., 2016)
- *not with* methamphetamine (Adhikary *et al*., 2017)

a.2. <u>*Restricted access…*</u>

a.2.1. and withdrawal followed by cue-induced relapse:

- cocaine (Hollander and Carelli, 2005)
- sucrose (Grimm *et al*., 2005)
- methamphetamine (Caprioli *et al*., 2015c)
- cocaine (Nugent *et al*., 2017), in mice
- sucrose (Nugent *et al.*, 2017), in mice
- nicotine (Funk *et al*., 2016)

a.2.2. *and extinction followed by cue-induced relapse:*

- cocaine (Nugent *et al*., 2017), in mice
- *not with* sucrose (Nugent *et al*., 2017), in mice

a.3. <u>*Intermittent access*</u> *and withdrawal followed by cue-induced relapse:*

- cocaine (James *et al*., 2018)

a.4. <u>*A single session*</u> *and withdrawal followed by cue-induced relapse:*

- cocaine (Halbout *et al*., 2014), in mice
- alcohol (Mijakowska *et al.*, 2017), in mice
- *not with* palatable food (Halbout *et al*., 2014), in mice

b. ***PASSIVE ADMINISTRATION…*** (non-contingent)

b.1. <u>*Conditioned place preference:*</u>

- morphine (Li *et al*., 2008)
- morphine (Hamed *et al*., 2012; USVs)
- morphine (Sun *et al*., 2017), in tree shrews
- saline (Hamed and Boguszewski, 2018; USVs)
- cocaine (Lubbers *et al*., 2016), in mice

b.2. <u>*Locomotor sensitization*</u>…

b.2.1. *induced by a non-contingent dose:*

- cocaine (Valjent *et al*., 2010; McCutcheon *et al*., 2011)
- methamphetamine (Wu *et al*., 2016)
- morphine (Valjent *et al*., 2010)

b.2.2. *induced by a non-contingent dose of cocaine:*

- *not with* sucrose (Grimm *et al*., 2006)

b.2.3. *induced by cues:*

- *not with* cocaine (Diehl *et al*., 2013)
- sucrose (Grimm *et al*., 2008; Harkness *et al*., 2010)

c. ***CONDITIONED FEAR*** (Pickens *et al.*, 2009)

As can be seen in the list, incubation of seeking is observed using rats, mice and even tree shrews, all close to our order (Figure 7).

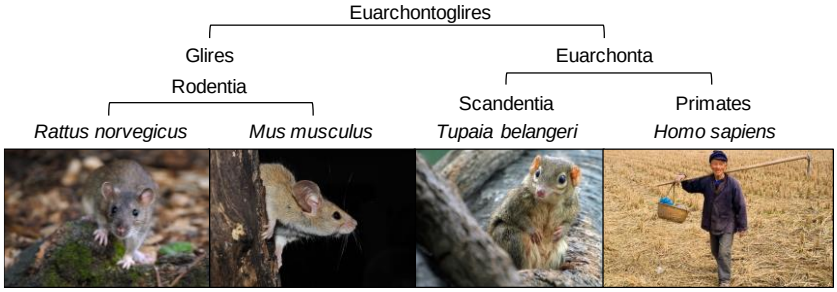


Figure 7. Phylogeny of the incubation of seeking. The phenomenon has been observed in at least four animal species, including humans, all of them phylogenetically close, belonging to:

>Animalia
>Chordata
>Vertebrata
>Mammalia
>Theria
>Eutheria
>Boreoeutheria
>Euarchontoglires >>>

It has been achieved after extended access protocols with a multitude of substances (Figure 8) but also after intermittent access, restricted access and after a single session of self-administration. Also when abstinence was a forced withdrawal, voluntary or even after extinction protocols. And inducing the seeking through exposure to cues, context or stress.

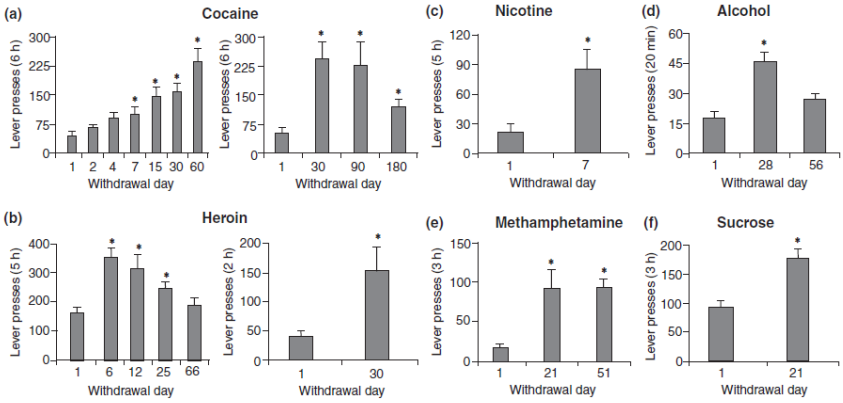


Figure 8. Incubation of seeking after extended access. The seeking increases along with forced abstinence of cocaine, nicotine, alcohol, heroin, methamphetamine and sucrose, among other substances (Pickens *et al*., 2011).

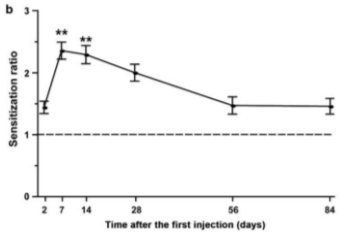


Figure 9. Incubation of locomotor sensitization. The ability of a cocaine injection to induce a locomotor activation increases as the days go by (Valjent *et al*., 2010).

In addition, incubation has not only been observed in paradigms of self-administration, but also in paradigms of place conditioning (CPP and CPA) and locomotor sensitization (Figure 9), both after non-contingent injections of the substance (or shocks in the case of CPA). Although it has not been possible to induce incubation with all the substances tested in all the paradigms, we think it is very likely that it is simply because the optimal experimental circumstances have not been achieved, just as restricted access sometimes induces incubation and sometimes does not .

There is a similar process in the pharmacological and toxicological literature: preconditioning or hormesis. A mild toxic insult induces a protective response in the organism against insults of the same nature, only observable during a time window that goes from days to weeks (Calabrese, 2016). The difference with the incubation of seeking (or the locomotor sensitization) is the measured parameter: in the case of hormesis we measure the capacity of a stimulus (an insult: a toxic, hypoxia) to generate damage while in the case of incubation we measure the capacity of a stimulus (cues associated with the substance, stress, an injection of the substance) to provoke a response (seeking or locomotor activity). Therefore, while in hormesis we observe a tissue response, in the incubation of seeking or locomotor sensitization we observe a behavior.

It is also possible that the incubation of the locomotor sensitization and of the seeking are independent phenomena, as well as the incubation of the CPP and CPA. However, we believe that the incubation of seeking induced by cues is less likely to have a different substrate depending on whether it is one substance or another.

1.2.3. Psychobiology of seeking incubation

Substances and research groups. Most of the studies of seeking incubation focus on a single substance. Only a few study two substances in parallel: cocaine and sucrose (Grimm *et al*., 2002; Shin *et al*., 2016; Swinford-Jackson *et al*., 2016) cocaine and palatable food (Halbout *et al*., 2014), methamphetamine and heroin (Theberge *et al*., 2013; Venniro *et al*., 2017b) and methamphetamine and palatable food (Krasnova *et al*., 2014). The remaining studies focus on one substance: two thirds study the cocaine seeking incubation, and the remaining third is divided between methamphetamine, heroin and sucrose, with anecdotal studies of morphine, nicotine, ethanol, saccharin and palatable food. The published reviews that totally or partially deal with the incubation phenomenon focus almost exclusively on the incubation of cocaine seeking, largely because it is the most studied substance (Dong *et al*., 2017; Li *et al*., 2015a; Li and Wolf, 2015; Loweth *et al*., 2014b; Lu *et al*., 2006, 2004; Marchant *et al*., 2013; Pickens *et al*., 2011; Wolf, 2016; Wolf and Ferrario, 2010; Wolf and Tseng, 2012). In addition, while it is true that the phenomenon has attracted more researchers over the years, most of the publications, including these reviews, belong to a handful of groups, most of them from the U.S.A.: the groups of Marina E. Wolf and Yavin Shaham, who have collaborated several times, of Karen K. Szumlinski and Jeffrey W. Grimm. The latter published with Yavin Shaham the first article on the incubation of seeking in rats. It was using cocaine. He then specialized in the incubation of sucrose seeking (Figure 10).

Marina E. Wolf *Yavin Shaham* *Jeffrey W. Grimm* *Karen K. Szumlinski*

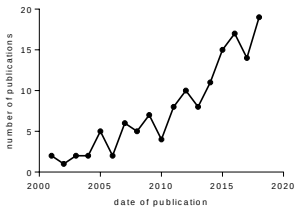


Figure 10. Publications about seeking incubation. Most come from a handful of US research groups, although as the number of annual publications increases, so does the number of groups involved.

Biochemical and pharmacological studies in seeking incubation. We can differentiate three types of studies:

- consequences of reacquisition, exposure to cues or extinction tests,
- consequences of pharmacological manipulation of a region, and
- biochemical changes throughout withdrawal.

A recurrent problem is the differential manipulation at short and long abstinence times (for example, the use of agonists of a pharmacological target at short times and of its antagonists at long times or vice versa), or even the absolute lack of manipulation at one of the times. The most studied brain region is undoubtedly the nucleus accumbens, followed by the prefrontal cortex and the amygdala. Other regions studied in several articles are the ventral tegmental area, the dorsal striatum and the hippocampus. There are also studies with systemic intraperitoneal (*i.p.*) and subcutaneous (*s.c.*) administrations.

The different results found in the literature are presented in tables, grouped according to the type of study: pharmacological manipulation of a region (Table 2), effect of the extinction test (Table 3) or changes in biochemical variables (Table 4). In the following section (*Involvement of brain regions in the incubation of seeking*) the main findings will be discussed, region by region, giving more importance to the manipulation studies, and secondarily to the studies of extinction tests and changes in biochemical variables. Then the effects of stress and environmental enrichment, of age and sex, as well as the kinetics of incubation will be presented. Finally, a summary of the state of the art will be outlined.

LEGEND FOR TABLES 2, 3 and 4

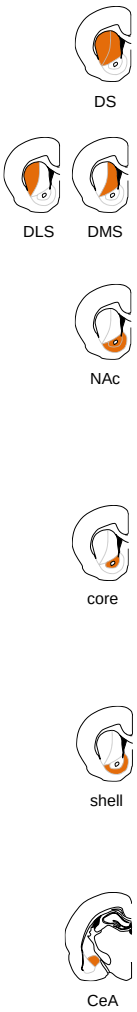
Shaded in grey those studies in which the same manipulation was performed at both times of abstinence.

↓, decrease; ↑, increase; ↔, changes in both directions; –, no effect; n.d., no determined; ( ), reinterpretation; ~~(inter)~~, no interaction in ANOVA; rdq, effect over readquisition; z, *vs* sucrose; *, *vs* short access.

S: substance; meth: methamphetamine; morph: morphine-CPP

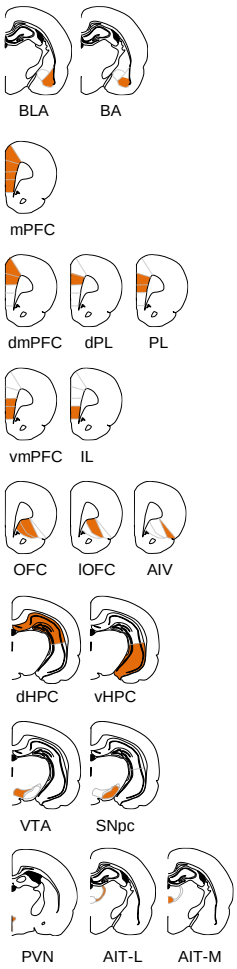
References published before / after the start of the present investigation (2011)

Table 2. Effects of different manipulations on seeking.



region	MANIPULATION	S	withdrawal early	withdrawal late	reference
DS	anti-β-endorphin	cocaine	–	n.d.	(Dikshtein *et al.*, 2013)
DS	β-endorphin	cocaine	n.d.	–	(Dikshtein *et al.*, 2013)
DS	GDNF, post last SA day	heroin	–	n.d.	(Airavaara *et al.*, 2011)
DLS	muscimol/baclofen (GABA agonists)	cocaine	↓	↓	(Pacchioni *et al.*, 2011)
DLS	SCH23390 (D1 antagonist)	meth	n.d.	↓	(Li *et al.*, 2015d)
DCS	SCH23390 (D1 antagonist)	meth	–	↓	(Li *et al.*, 2015d)
DMS	SCH23390 (D1 antagonist)	meth	n.d.	↓	(Li *et al.*, 2015d)
DMS	SCH39166 (D1 antagonist)	meth	–	↓	(Caprioli *et al.*, 2017)
DMS	raclopride (D2 antagonist)	meth	–	↓	(Caprioli *et al.*, 2017)
DMS	SCH23390 (D1 antagonist), unilateral	meth	n.d.	–	(Li *et al.*, 2018)
DMS_{cues}	inactivation with Daun02	meth	n.d.	↓	(Caprioli *et al.*, 2017)
DMS<AIT-L	SCH23390 < muscimol/baclofen, ipsi/contra	meth	–	↓	(Li *et al.*, 2018)
NAc	β-endorphin	cocaine	n.d.	↓	(Dikshtein *et al.*, 2013)
NAc	anti-β-endorphin	cocaine	↑	n.d.	(Dikshtein *et al.*, 2013)
NAc	epigenetic targets	cocaine	n.d.	↔	(Massart *et al.*, 2015)
NAc	GDNF, post last SA day	heroin	↑	–	(Airavaara *et al.*, 2011)
NAc	anti-GDNF, after SA	heroin	n.d.	–	(Airavaara *et al.*, 2011)
NAc	epigenetic targets	sucrose	n.d.	↔	(Massart *et al.*, 2015)
NAc	naltrindol (DOR antagonist) + β-endorphin	cocaine	n.d.	–	(Dikshtein *et al.*, 2013)
NAc	CTAP (MOR antagonist) + β-endorphin	cocaine	n.d.	↓	(Dikshtein *et al.*, 2013)
NAc	naltrindol (DOR antagonist)	cocaine	↑(~~inter~~)	–	(Dikshtein *et al.*, 2013)
NAc	CTAP (MOR antagonist)	cocaine	–	–	(Dikshtein *et al.*, 2013)
core	PAM-mGluR1	cocaine	n.d.	↓	(Loweth *et al.*, 2014a)
core	Naspm (CP-AMPAR antagonist)	cocaine	–	↓	(Conrad *et al.*, 2008)
core	rolipram (PDE-type IV antagonist)	heroin	n.d.	↓	(Sun *et al.*, 2015)
core	SB-277011A (D3 antagonist)	cocaine	n.d.	↓	(Xi *et al.*, 2013)
core	*TrkB* siRNA	cocaine	↑(~~inter~~)	–	(Li *et al.*, 2013b)
core	Naspm (CP-AMPAR antagonist)	meth	n.d.	↓	(Scheyer *et al.*, 2016)
core	SCH23390 (D1 antagonist)	sucrose	↓	↓	(Grimm *et al.*, 2011)
core	overexpression of Homer	cocaine	–	–	(Loweth *et al.*, 2014a)
core<PL	CI-AMPAR resilencing	cocaine	n.d.	↓	(Ma *et al.*, 2014)
shell	SB-277011A (D3 antagonist)	cocaine	n.d.	↓	(Xi *et al.*, 2013)
shell	*TrkB* siRNA	cocaine	–	↓(~~inter~~)	(Li *et al.*, 2013b)
shell	SCH23390 (D1 antagonist)	sucrose	↓	↓	(Grimm *et al.*, 2011)
shell	*Grin2b* or *Sk2* iRNA	cocaine	–	↓	(Wang *et al.*, 2018)
shell	*Dnmt3a2* shRNA	cocaine	↓	↓(~~inter~~)	(Cannella *et al.*, 2018)
shell<BA	CP-AMPAR resilencing	cocaine	n.d.	↓	(Lee *et al.*, 2013)
shell<IL	optogenetic activation	cocaine	n.d.	↓	(Müller Ewald *et al.*, 2018)
shell<IL	CP-AMPAR resilencing	cocaine	n.d.	↑	(Ma *et al.*, 2014)
CeA	U0126 (MEK1/2 inhibitor)	cocaine	n.d.	↓	(Lu *et al.*, 2005b)
CeA	AP-5 (NMDAR antagonist)	cocaine	↓	n.d.	(Lu *et al.*, 2005b)
CeA	LY379268 (mGluR2/3 agonist)	cocaine	–	↓	(Lu *et al.*, 2007)
CeA	SB-277011A (D3 antagonist)	cocaine	n.d.	↓	(Xi *et al.*, 2013)
CeA	muscimol/baclofen (GABA agonists)	meth	–	↓	(Li *et al.*, 2015b)
CeA	U0126 (MEK1/2 inhibitor)	morph	n.d.	↓	(Li *et al.*, 2008)
CeA	NMDA	morph	↑	n.d.	(Li *et al.*, 2008)
CeA	LY379268 (mGluR2/3 agonist)	sucrose	–	↓	(Uejima *et al.*, 2007)
CeA	SCH39166 (D1 antagonist)	meth	n.d.	↓	(Venniro *et al.*, 2017a)
CeA	raclopride (D2 antagonist)	meth	n.d.	–	(Venniro *et al.*, 2017a)
CeA<AIV	inactivation (DREADD)	meth	n.d.	↓	(Venniro *et al.*, 2017a)
CeA_{claves}	inactivation with Daun02	nicotine	n.d.	↓	(Funk *et al.*, 2016)

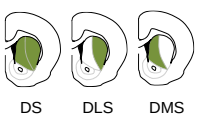
region	MANIPULATION	S	withdrawal early	withdrawal late	reference
BA	U0126 (MEK1/2 inhibitor)	cocaine	n.d.	–	(Lu *et al.*, 2005b)
BA	LY379268 (mGluR2/3 agonist)	cocaine	n.d.	–	(Lu *et al.*, 2007)
BA	muscimol/baclofen (GABA agonists)	meth	–	–	(Li *et al.*, 2015b)
BLA	SB-277011A (D3 antagonist)	cocaine	n.d.	–	(Xi *et al.*, 2013)
BLA	U0126 (MEK1/2 inhibitor)	morph	n.d.	–	(Li *et al.*, 2008)
BLA	SCH39166 (D1 antagonist)	meth	n.d.	–	(Venniro *et al.*, 2017a)
mPFC	WAY163909 (5HT2C agonist)	cocaine	↓↓	↓	(Swinford-Jackson *et al.*, 2016)
dmPFC	MPEP, PTEP, JNJ (mGluRI antagonists)	cocaine	–	n.d.	(Ben-Shahar *et al.*, 2013)
dmPFC	induction of brevican in KO$^{\pm}$ (CPP)	cocaine	–	–	(Lubbers *et al.*, 2016)
dmPFC	bicuculline/saclofen (GABA antagonists)	cocaine	–	n.d.	(Koya *et al.*, 2009)
dmPFC	muscimol/baclofen (GABA agonists)	cocaine	n.d.	–	(Koya *et al.*, 2009)
dPL	muscimol/baclofen (GABA agonists)	meth	n.d.	–	(Li *et al.*, 2015b)
PL	wortmannin (PI3K inhibitor)	cocaine	n.d.	↓	(Szumlinski *et al.*, 2018)
vmPFC	bicuculline/saclofen (GABA antagonists)	cocaine	↑	n.d.	(Koya *et al.*, 2009)
vmPFC	muscimol/baclofen (GABA agonists)	cocaine	n.d.	↓	(Koya *et al.*, 2009)
vmPFC	MPEP, PTEP, JNJ (mGluRI antagonists)	cocaine	–	n.d.	(Ben-Shahar *et al.*, 2013)
vmPFC	DHPG (mGluRI agonist)	cocaine	n.d.	↓2nd test	(Ben-Shahar *et al.*, 2013)
vmPFC	repression of *Homer*	cocaine	–	–	(Gould *et al.*, 2015)
vmPFC	TAT peptide (PKC antagonist)	cocaine	n.d.	↓	(Miller *et al.*, 2017)
IL	optogenetic activation	cocaine	n.d.	–	(Müller Ewald *et al.*, 2018)
IL	muscimol/baclofen (GABA agonists)	meth	–	–	(Li *et al.*, 2015b)
OFC	muscimol/baclofen (GABA agonists)	heroin	–(↓)	↓ (~~inter~~)	(Fanous *et al.*, 2012)
OFC$_{claves}$	inactivation with Daun02	nicotine	n.d.	–	(Funk *et al.*, 2016)
OFC$_{claves}$	inactivation with Daun02	heroin	n.d.	↓	(Fanous *et al.*, 2012)
lOFC	muscimol/baclofen (GABA agonists)	meth	n.d.	–	(Li *et al.*, 2015b)
lOFC	muscimol/baclofen (GABA agonists)	meth	n.d.	–	(Venniro *et al.*, 2017a)
AIV	muscimol/baclofen (GABA agonists)	meth	n.d.	–	(Venniro *et al.*, 2017a)
dHPC	induction of brevican in KO$^{\pm}$ (CPP)	cocaine	–	↓	(Lubbers *et al.*, 2016)
vHPC	lesion	cocaine	–	–	(Karlsson *et al.*, 2013)
VTA	GDNF, post last SA day	cocaine	↑	↑	(Lu *et al.*, 2009)
VTA	U0126 (MEK1/2 inhibitor), after last SA	cocaine	–	–	(Lu *et al.*, 2009)
VTA	mRNA *Gdnf*, during wd	cocaine	↑	↑↑	(Lu *et al.*, 2009)
VTA	anti-GDNF, during wd	cocaine	↓	↓↓	(Lu *et al.*, 2009)
VTA	*Grin1*-KO in DAT+ cells	cocaine	n.d.	↓	(Mameli *et al.*, 2009)
VTA	GDNF, post last SA day	heroin	–	–	(Airavaara *et al.*, 2011)
VTA	anti-GDNF, during wd	heroin	n.d.	–	(Airavaara *et al.*, 2011)
SNpc	GDNF, post last SA day	cocaine	–	–	(Lu *et al.*, 2009)
AIT-L (Th)	muscimol/baclofen, bilateral	meth	n.d.	↓	(Li *et al.*, 2018)
AIT-L (Th)	muscimol/baclofen, unilateral	meth	n.d.	–	(Li *et al.*, 2018)
AIT-M (Th)	muscimol/baclofen, bilateral	meth	n.d.	–	(Li *et al.*, 2018)
PVN (HPT)	shNMUR2	food	↓,–rdq	↓,↓rdq	(McCue *et al.*, 2018)

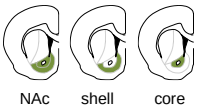


route	MANIPULATION	S	withdrawal early	withdrawal late	reference
i.p.	SCH39166 (D1 antagonist)	sucrose	↓	↓	(Grimm *et al.*, 2011)
i.p.	LY379268 (mGluR2/3 agonist)	cocaine	–	↓	(Lu *et al.*, 2007)
i.p.	LY379268 (mGluR2/3 agonist)	sucrose	–	↓	(Uejima *et al.*, 2007)
i.p.	SCH39166 (D1 antagonist)	meth	n.d.	↓	(Caprioli *et al.*, 2017)
i.p.	aripiprazole (D2 partial agonist)	cocaine	n.d.	–	(Madsen *et al.*, 2017)
i.p.	PAM-mGluR1	meth	n.d.	↓	(Scheyer *et al.*, 2016)
i.p.	A-841720 (mGluR1 antagonist)	cocaine	n.d.	↑	(Halbout *et al.*, 2014)
i.p.	quinpirole (D2 agonist)	sucrose	–	–	(Glueck *et al.*, 2017)
i.p.	SB-277011A (D3 antagonist)	cocaine	↓	↓	(Xi *et al.*, 2013)
i.p.	naltrexone (MOR>KOR antagonist), -1 day	heroin	↑	–	(Zhou *et al.*, 2009)
i.p.	naloxone (opioidergic antagonist)	heroin	–(↓)	↓ (~~inter~~)	(Theberge *et al.*, 2012)
s.c.	PAM-mGluR2	meth	–	↓	(Caprioli *et al.*, 2015a)
s.c.	SKF81297 (D1 agonist)	sucrose	–	–	(Glueck *et al.*, 2017)
s.c.	(+)-naltrexone (TLR4 antagonist), during wd	heroin	n.d.	↓	(Theberge *et al.*, 2013)
s.c.	(+)-naltrexone (TLR4 antagonist), during wd	meth	n.d.	–	(Theberge *et al.*, 2013)
s.c./i.p.	(+)-naltrexone (TLR4 antagonist)	heroin	n.d.	–	(Theberge *et al.*, 2013)

Table 3. Effects of extinction test on certain biochemical parameters.

region	EXTINCTION TEST EFFECT	S	early	late	reference
DS	5-HT, 5-HIAA	morph	–	↑	(Hamed & Boguszewski, 2018)
DLS	NSF, proteolytic activity	cocaine	–	↑	(Werner *et al.*, 2015)
DLS	Ubiquitinated proteins	cocaine	↑	–	(Werner *et al.*, 2015)
DLS	gene expression	meth	↔	↔	(Li *et al.*, 2015d)
DLS	*Fos*	sucrose	↑	↑↑	(Grimm *et al.*, 2016)
DLS	Fos (%D1·MSN=%D2·MSN)	meth	–	–	(Caprioli *et al.*, 2017)
DLS$_{cues}$	gene expression	meth	↔	↔	(Li *et al.*, 2015d)
DMS	Fos (%D1·MSN=%D2·MSN)	meth	–	↑	(Caprioli *et al.*, 2017)

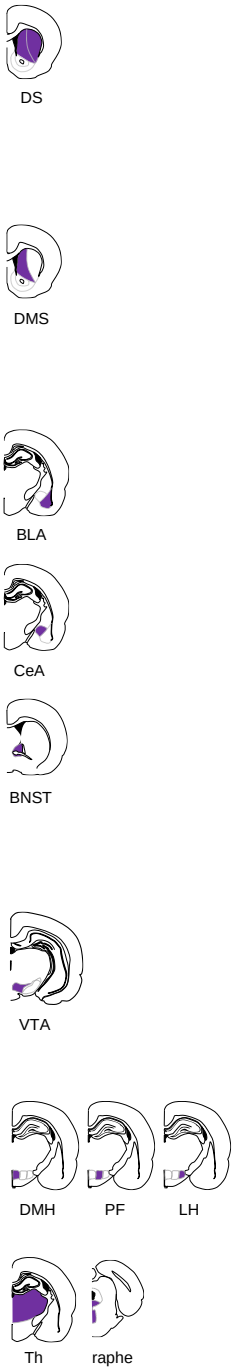


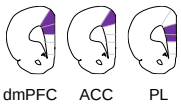


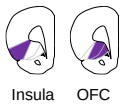
region	EXTINCTION TEST EFFECT	S	withdrawal early	withdrawal late	reference
NAc	β-endorphin	cocaine	↑	–	(Dikshtein *et al.*, 2013)
NAc	proteolytic activity	cocaine	↑	–	(Werner *et al.*, 2015)
NAc	DNA methylation	cocaine	↑	↑↑	(Massart *et al.*, 2015)
NAc	pERK and pCREB	heroin	–	↓	(Sun *et al.*, 2015)
NAc	Ala, Gln, Gly, MHPG	morph	–	↓	(Hamed & Boguszewski, 2018)
core	*Fos*	sucrose	↑	↑↑	(Grimm *et al.*, 2016)
core	*Fos*	nicotine	–	↑	(Funk *et al.*, 2016)
core	BDNF	cocaine	–	↑	(Gueye *et al.*, 2018)
shell	*Fos*	sucrose	↑	↑↑	(Grimm *et al.*, 2016)
shell	*Fos*	nicotine	–	↑	(Funk *et al.*, 2016)
BNST	*Fos*	sucrose	–	–	(Grimm *et al.*, 2016)
Amg	trypsine	coca	–	↓	(Werner *et al.*, 2015)
Amg	5-HT, 5-HIAA, Glu	morph	–	↑	(Hamed & Boguszewski, 2018)
CeA	pERK	cocaine	–	↑	(Lu *et al.*, 2005b)
CeA	pERK	cocaine	–	↑	(Thiel *et al.*, 2012)
CeA	pERK	morph	–	↑	(Li *et al.*, 2008)
CeA	*Fos*	sucrose	–	↑	(Grimm *et al.*, 2016)
CeA	*Fos*	nicotine	–	↑	(Funk *et al.*, 2016)
CeA	*Fos*	meth	n.d.	↑	(Venniro *et al.*, 2017a)
CeC/L	c-Fos, p-Jun, pERK	alcohol	n.d.	↑	(Radwanska *et al.*, 2008)
CeM, MePD	c-Fos, p-Jun, pERK	alcohol	n.d.	–	(Radwanska *et al.*, 2008)
BLA	pERK	cocaine	–	–	(Lu *et al.*, 2005b)
BLA	pERK, pCREB	morph	–	–	(Li *et al.*, 2008)
BLA	*Fos*	sucrose	–	–	(Grimm *et al.*, 2016)
BLA	*Fos*	meth	n.d.	↑	(Li *et al.*, 2018)
BLA	*Fos*	nicotine	–	↑	(Funk *et al.*, 2016)
BLA	*Fos*	meth	n.d.	–	(Venniro *et al.*, 2017a)
LaD	c-Fos, p-Jun, pERK	alcohol	n.d.	–	(Radwanska *et al.*, 2008)
BA, LaV, BMP	c-Fos, p-Jun, pERK	alcohol	n.d.	↑	(Radwanska *et al.*, 2008)
mPFC	endocitosis de 5HT2C	cocaine	n.d.	↑	(Swinford-Jackson *et al.*, 2016)
mPFC	proteolytic activity	cocaine	↑↑	↑	(Werner *et al.*, 2015)
mPFC	5-HT, DA	morph	–	↑	(Hamed & Boguszewski, 2018)
mPFC	Gln, Ala, GABA, Tau	morph	–	↓	(Hamed & Boguszewski, 2018)
dmPFC	activated neurons genes	heroin	n.d.	↔	(Fanous *et al.*, 2013)
dmPFC	pERK	cocaine	–	↑	(Koya *et al.*, 2009)
dmPFC	mGluR1/5	cocaine	–	–	(Ben-Shahar *et al.*, 2013)
dmPFC	pPKCε	cocaine	–	–	(Miller *et al.*, 2017)
dmPFC	*Fos*	meth	n.d.	↑	(Li *et al.*, 2018)
dmPFC	*Fos*	nicotine	–	↑	(Funk *et al.*, 2016)
PL	number of activated neurons	cocaine	–	↑	(West *et al.*, 2014)
PL	BDNF	cocaine	–	↑	(Gueye *et al.*, 2018)
PL	*Fos*	sucrose	–	↑	(Grimm *et al.*, 2016)
ACC	*Fos*	sucrose	–	↑	(Grimm *et al.*, 2016)
vmPFC	activated neurons genes	heroin	n.d.	↔	(Fanous *et al.*, 2013)
vmPFC	pERK	cocaine	–	↑	(Koya *et al.*, 2009)
vmPFC	DA release	cocaine	–	↓	(Shin *et al.*, 2016)
vmPFC	Glu release	cocaine	–	↑	(Shin *et al.*, 2016)
vmPFC	mGluR1/5	cocaine	–	↓	(Ben-Shahar *et al.*, 2013)
vmPFC	pPKCε	cocaine	↓	↑	(Miller *et al.*, 2017)
vmPFC	pAKT/AKT	cocaine	↑	↑	(Szumlinski *et al.*, 2018)
vmPFC	DA and Glu release	sucrose	–	–	(Shin *et al.*, 2016)
vmPFC	*Fos*	meth	n.d.	↑	(Li *et al.*, 2018)
vmPFC	*Fos*	nicotine	–	↑	(Funk *et al.*, 2016)
IL	number of activated neurons	cocaine	–	–	(West *et al.*, 2014)
IL	*Fos*	sucrose	–	↑	(Grimm *et al.*, 2016)
OFC	activated neurons genes	heroin	n.d.	↔	(Fanous *et al.*, 2013)
OFC	*Fos*	sucrose	–	–	(Grimm *et al.*, 2016)
OFC	*Fos*	heroin	– (↑)	↑ (~~inter~~)	(Fanous *et al.*, 2012)
OFC	*Fos*	nicotine	–	↑	(Funk *et al.*, 2016)
AI	*Fos*	meth	n.d.	↑	(Li *et al.*, 2018)
SS	*Fos*	sucrose	–	↑	(Grimm *et al.*, 2016)
HPC	5-HT, 5-HIAA, Tau	morph	–	↑	(Hamed & Boguszewski, 2018)
dHPC	*Fos*	sucrose	–	–	(Grimm *et al.*, 2016)
vSub	*Fos*	sucrose	–	–	(Grimm *et al.*, 2016)
VTA	*Fos*	sucrose	–	–	(Grimm *et al.*, 2016)
VTA	BDNF	cocaine	–	↑	(Gueye *et al.*, 2018)
VTA	Ala, GABA, Tau	morph	–	↓	(Hamed & Boguszewski, 2018)
SNpc	*Fos*	sucrose	–	–	(Grimm *et al.*, 2016)
SNpr	*Fos*	sucrose	–	–	(Grimm *et al.*, 2016)
AIT-L (Th)	*Fos*	meth	n.d.	↑	(Li *et al.*, 2018)
AIT-L>DMS	*Fos*	meth	n.d.	↑	(Li *et al.*, 2018)
AIT-M (Th)	*Fos*	meth	n.d.	–	(Li *et al.*, 2018)
Pf (Th)	*Fos*	meth	n.d.	–	(Li *et al.*, 2018)

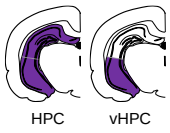
Table 4. Biochemical changes during withdrawal. The vast majority of the studies do not analyse the interaction effects between the consumption and the duration of abstinence.

region	BIOCHEMICAL PARAMETER	S	withdrawal early	withdrawal late	reference
DS	rCBV	cocaine	n.d.	↓	(Gozzi *et al.*, 2011)
DS	bCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
DS	AMPA, NMDA (binding); *Grm1*	1d-cocaine	–	–	(Halbout *et al.*, 2014)
DS	MeCP2, BDNF, TrkB (WB, qPCR); *Oprm1*	heroin	–	–	(Theberge *et al.*, 2012)
DS	*Gria1/2/3*, *Grin1/2a/2b*, *Grm1/5*	meth	–	–	(Li *et al.*, 2015d)
DS	*Bdnf*, *Trkb*, *Sirt1*, *Sirt2*, *Crebbp*, *Suv39n1*	meth	–	–	(Li *et al.*, 2015d)
DS	*G9a*, *Glp*, *Kdm1a*, *Dnmt3a*, *Hdac1-5*	meth	–	–	(Li *et al.*, 2015d)
DS	*Mll1* (H3K4 methyltrasferase)	meth	–	↑	(Li *et al.*, 2015d)
DS	OPRM1	oxycodone	n.d.	↓	(Blackwood *et al.*, 2018)
DS	OPRD1, OPRK1, *Oprm1*, *Oprd1*, *Oprk1*	oxycodone	n.d.	–	(Blackwood *et al.*, 2018)
DMS	DAT (autoradiography)	cocaine	n.d.	–	(Ben-Shahar *et al.*, 2006)
DMS	D1, D2, NMDA (autoradiography)	cocaine	–	–	(Ben-Shahar *et al.*, 2007)
DMS	*Htr1b* (ISH)	cocaine	–	↓	(Neumaier *et al.*, 2009)
DMS	*Htr6* (ISH)	cocaine	–	–	(Neumaier *et al.*, 2009)
Amg	bCBV, rCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
Amg	TH, DAT	cocaine	–z	–z	(Grimm *et al.*, 2002)
Amg	BDNF	cocaine	–	↑	(Grimm *et al.*, 2003)
Amg	NGF	cocaine	–	–	(Grimm *et al.*, 2003)
Amg	BDNF, NGF	sucrose	–	–	(Grimm *et al.*, 2003)
BLA	GluA1	cocaine	↑	↑	(Lu *et al.*, 2005a)
BLA	GluA2, NR1	cocaine	–	–	(Lu *et al.*, 2005a)
BLA	NR2A	cocaine	↑	–	(Lu *et al.*, 2005a)
BLA	NR2B	cocaine	–	↓	(Lu *et al.*, 2005a)
BLA	AMPA (binding); *Grm1*	1d-cocaine	–	–	(Halbout *et al.*, 2014)
BLA	NMDA (binding)	1d-cocaine	–	↓	(Halbout *et al.*, 2014)
CeA	GluA2	cocaine	↑	↑	(Lu *et al.*, 2005a)
CeA	NR1	cocaine	–	↑	(Lu *et al.*, 2005a)
CeA	NR2A, NR2B, GluA1	cocaine	–	–	(Lu *et al.*, 2005a)
CeA	AMPA (binding); *Gmr1*	1d-cocaine	–	–	(Halbout *et al.*, 2014)
CeA	NMDA (binding)	1d-cocaine	–	↓	(Halbout *et al.*, 2014)
CeA	RNA-Seq	meth	↔	↔	(Cates *et al.*, 2018)
CeA	*Crh* (ISH)	heroin	–	–	(Shalev *et al.*, 2001)
v/dBNST	*Crh* (ISH)	heroin	–	–	(Shalev *et al.*, 2001)
VTA	DAT (autoradiography)	cocaine	n.d.	–	(Ben-Shahar *et al.*, 2006)
VTA	D1, D2, NMDA (autoradiography)	cocaine	–	–	(Ben-Shahar *et al.*, 2007)
VTA	mGluR1, mGluR5, Homer1, Homer2, NR2A,	cocaine	–	–	(Ben-Shahar *et al.*, 2009)
VTA	GluA1, GluA2	cocaine	–	–	(Conrad *et al.*, 2008)
VTA	GluA3	cocaine	–	↑	(Conrad *et al.*, 2008)
VTA	GluA1, mGluR5, PSD95, β-act, PICK1, Homer1	cocaine	n.d.	–	(Ghasemzadeh *et al.*, 2011)
VTA	NR1	cocaine	n.d.	↓	(Ghasemzadeh *et al.*, 2011)
VTA	BDNF	cocaine	–	↑	(Grimm *et al.*, 2003)
VTA	NGF	cocaine	–	–	(Grimm *et al.*, 2003)
VTA	GluA1, PKA, AC	cocaine	–z	–z	(Lu *et al.*, 2003)
VTA	GluA2, TH, Cdk5	cocaine	↑z	–z	(Lu *et al.*, 2003)
VTA	NR1	cocaine	↑z	↑z	(Lu *et al.*, 2003)
VTA	BDNF	cocaine	–	–	(Thiel *et al.*, 2012)
VTA	AMPA (binding)	1d-cocaine	↓	↓	(Halbout *et al.*, 2014)
VTA	NMDA (binding); *Grm1*	1d-cocaine	–	–	(Halbout *et al.*, 2014)
VTA	*Gdnf*	heroin	↓	–	(Airavaara *et al.*, 2011)
VTA	GDNF	heroin	–	–	(Airavaara *et al.*, 2011)
VTA	BDNF, NGF	sucrose	–	–	(Grimm *et al.*, 2003)
HPT	bCBV, rCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
DMH/PF	orexin$^+$ neurons	cocaine-IntA	↑	–	(James *et al.*, 2018)
DMH/PF	MCH$^+$ neurons	cocaine-IntA	–	–	(James *et al.*, 2018)
LH	orexin$^+$ neurons	cocaine-IntA	↑	↑	(James *et al.*, 2018)
LH	MCH$^+$ neurons	cocaine-IntA	–	–	(James *et al.*, 2018)
raphe	bCBV	cocaine	n.d.	↓	(Gozzi *et al.*, 2011)
raphe	rCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
Th	rCBV	cocaine	n.d.	↓	(Gozzi *et al.*, 2011)
Th	bCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)









region	BIOCHEMICAL PARAMETER	S	withdrawal early	withdrawal late	reference
PFC	MeCP2, BDNF, TrkB (WB, qPCR); *Oprm1*	heroin	–	–	(Theberge *et al.*, 2012)
mPFC	DAT (autoradiography)	cocaine	n.d.	–	(Ben-Shahar *et al.*, 2006)
mPFC	D1, D2, NMDA (autoradiography)	cocaine	–	–	(Ben-Shahar *et al.*, 2007)
mPFC	mGluR1, mGluR5	cocaine	–	–	(Ben-Shahar *et al.*, 2009)
mPFC	Homer1b/c	cocaine	↑	–	(Ben-Shahar *et al.*, 2009)
mPFC	Homer2a/b	cocaine	–	–	(Ben-Shahar *et al.*, 2009)
mPFC	NR2A, NR2B	cocaine	–	↑	(Ben-Shahar *et al.*, 2009)
mPFC	*Cfos*, *Egr1*, *Arc*, *Nr4a1*	cocaine	↓	↓	(Freeman *et al.*, 2008)
mPFC	*Cart*, *Npy*	cocaine	↑	–	(Freeman *et al.*, 2008)
mPFC	*Gria1*, *Gria2*, *Grin1*, *Homer1/2*, *Cdk5*	cocaine	–	–	(Freeman *et al.*, 2008)
mPFC	*Nefl*, *Cd47*, *Drd5*, *Adora2b* (microarray)	cocaine	↔	↔	(Freeman *et al.*, 2010)
mPFC	bCBV	cocaine	n.d.	↓	(Gozzi *et al.*, 2011)
mPFC	rCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
mPFC	TH, DAT	cocaine	↑z	↑z	(Grimm *et al.*, 2002)
mPFC	proteomic (2D)	cocaine	↔	↔	(Lull *et al.*, 2009)
mPFC	5HT2C	cocaine	–	↓	(Swinford-Jackson *et al.*, 2016)
mPFC	DARPP-34 total, pThr34, pThr75	nicotine	–	–	(Abdolahi *et al.*, 2010)
mPFC	5HT2C	sucrose	–	–	(Swinford-Jackson *et al.*, 2016)
dmPFC	mGluR1, mGluR5	cocaine	–	–	(Ben-Shahar *et al.*, 2013)
dmPFC	GluA1, NR1, mGluR5, PSD95, β-act, PICK1, Homer1	cocaine	n.d.	–	(Ghasemzadeh *et al.*, 2011)
dmPFC	PI3K, p(Tyr)p85α	cocaine	–	–	(Szumlinski *et al.*, 2018)
dmPFC	Homer1, Homer2	cocaine	–	–	(Gould *et al.*, 2015)
dmPFC	pPKCε	cocaine	–	–	(Miller *et al.*, 2017)
ACC	GluA1, GluA2, GluA3	cocaine	n.d.	–	(Conrad *et al.*, 2008)
ACC	bCBV	cocaine	n.d.	↓	(Gozzi *et al.*, 2011)
ACC	rCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
ACC	AMPA (binding)	1d-cocaine	↓	–	(Halbout *et al.*, 2014)
ACC	NMDA (binding); *Grm1*	1d-cocaine	–	–	(Halbout *et al.*, 2014)
PL	AMPA (binding)	1d-cocaine	↓	–	(Halbout *et al.*, 2014)
PL	NMDA (binding)	1d-cocaine	–	–	(Halbout *et al.*, 2014)
PL	*Grm1*	1d-cocaine	–	↑	(Halbout *et al.*, 2014)
vmPFC	mGluR1, mGluR5	cocaine	–	–	(Ben-Shahar *et al.*, 2013)
vmPFC	GluA1, NR1, mGluR5, PSD95, β-act, PICK1, Homer1	cocaine	n.d.	–	(Ghasemzadeh *et al.*, 2011)
vmPFC	AKT, pAKT, PI3K, p(Tyr)p85α	cocaine	–	–	(Szumlinski *et al.*, 2018)
vmPFC	Homer1	cocaine	↑	–	(Gould *et al.*, 2015)
vmPFC	Homer2	cocaine	↑	↑	(Gould *et al.*, 2015)
vmPFC	pPKCε	cocaine	–	–	(Miller *et al.*, 2017)
IL	AMPA (binding)	1d-cocaine	↓	–	(Halbout *et al.*, 2014)
IL	NMDA (binding)	1d-cocaine	↓	↓	(Halbout *et al.*, 2014)
IL	*Grm1*	1d-cocaine	–	↑	(Halbout *et al.*, 2014)
OFC	bCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
OFC	rCBV	cocaine	n.d.	↓	(Gozzi *et al.*, 2011)
OFC	TH, DAT	cocaine	–	–	(Grimm *et al.*, 2002)
OFC	AMPA, NMDA (binding)	1d-cocaine	–	–	(Halbout *et al.*, 2014)
OFC	*Grm1*	1d-cocaine	↑	↑	(Halbout *et al.*, 2014)
OFC	RNA-Seq	meth	↔	↔	(Cates *et al.*, 2018)
SS	bCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
SS	rCBV	cocaine	n.d.	↓	(Gozzi *et al.*, 2011)
Insula	DARPP-34 pThr34	nicotine	–	↑	(Abdolahi *et al.*, 2010)
Insula	DARPP-34 pThr75	nicotine	↑	↑	(Abdolahi *et al.*, 2010)
Insula	DARPP-34	nicotine	–	–	(Abdolahi *et al.*, 2010)
M	bCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
M	rCBV	cocaine	n.d.	↓	(Gozzi *et al.*, 2011)
RS	bCBV, rCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
HPC	OPRM1, OPRD1, OPRK1, *Oprd1*	oxycodone	n.d.	–	(Blackwood *et al.*, 2018)
HPC	*Oprm1*	oxycodone	n.d.	↓	(Blackwood *et al.*, 2018)
HPC	*Oprk1*	oxycodone	n.d.	↓*	(Blackwood *et al.*, 2018)
vHPC	bCBV	cocaine	n.d.	↑	(Gozzi *et al.*, 2011)
vHPC	rCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
vHPC	BDNF	cocaine	–	–	(Thiel *et al.*, 2012)
dHPC	BDNF	cocaine	–	–	(Thiel *et al.*, 2012)
dCA1	AMPA (binding)	1d-cocaine	–	–	(Halbout *et al.*, 2014)
dCA1	NMDA (binding)	1d-cocaine	–	↓	(Halbout *et al.*, 2014)
dCA1	*Grm1*	1d-cocaine	↓	–	(Halbout *et al.*, 2014)
dCA3	AMPA (binding)	1d-cocaine	↓	–	(Halbout *et al.*, 2014)
dCA3	NMDA (binding)	1d-cocaine	–	↓	(Halbout *et al.*, 2014)
dCA3	*Grm1*	1d-cocaine	↓	↑	(Halbout *et al.*, 2014)
dDG	AMPA (binding)	1d-cocaine	↓	–	(Halbout *et al.*, 2014)
dDG	NMDA (binding)	1d-cocaine	–	↓	(Halbout *et al.*, 2014)
dDG	*Grm1*	1d-cocaine	–	–	(Halbout *et al.*, 2014)

region	BIOCHEMICAL PARAMETER	S	withdrawal early	withdrawal late	reference
NAc	GluA1	cocaine	↓	↑	(Conrad *et al.*, 2008)
NAc	GluA2	cocaine	↑	–	(Conrad *et al.*, 2008)
NAc	GluA3	cocaine	↑	↑	(Conrad *et al.*, 2008)
NAc	NR1, NR2A, NR2B	cocaine	–	–	(Conrad *et al.*, 2008)
NAc	pCaMKI, %pERK1	cocaine	–	–	(Ferrario *et al.*, 2011)
NAc	%pCaMKIIα/β	cocaine	–	↑	(Ferrario *et al.*, 2011)
NAc	%pERK2	cocaine	↑	↑	(Ferrario *et al.*, 2011)
NAc	GluA1, GluA1 pSer845, rectif.index, TARP-γ4	cocaine	n.d.	↑	(Ferrario *et al.*, 2011)
NAc	GluA3	cocaine	n.d.	↓	(Ferrario *et al.*, 2011)
NAc	GluA2, TARP-γ2	cocaine	n.d.	–	(Ferrario *et al.*, 2011)
NAc	NR1, NR2A, NR2B	cocaine	n.d.	–	(Ferrario *et al.*, 2012)
NAc	*Cfos*, *Egr1*	cocaine	↓	↓	(Freeman *et al.*, 2008)
NAc	*Arc*, *Nr4a1*	cocaine	–	↓(wd100)	(Freeman *et al.*, 2008)
NAc	*Cart*, *Npy*	cocaine	–	–	(Freeman *et al.*, 2008)
NAc	*Gria1*, *Gria2*, *Grin1*, *Homer1/2*, *Cdk5*	cocaine	–	–	(Freeman *et al.*, 2008)
NAc	*Egr2*	cocaine	–	↓	(Freeman *et al.*, 2010)
NAc	*Htr1d*	cocaine	↓	–	(Freeman *et al.*, 2010)
NAc	TH, DAT	cocaine	–z	–z	(Grimm *et al.*, 2002)
NAc	NGF	cocaine	–z	–z	(Grimm *et al.*, 2003)
NAc	BDNF	cocaine	–z	↑z	(Grimm *et al.*, 2003)
NAc	mGluR1, mGluR5	cocaine	–	↓	(Loweth *et al.*, 2014a)
NAc	GluA1, GluA2, NR1, PKA	cocaine	↑z	↑z	(Lu *et al.*, 2003)
NAc	TH, Cdk5, AC	cocaine	–z	–z	(Lu *et al.*, 2003)
NAc	BDNF	cocaine	–	–	(Thiel *et al.*, 2012)
NAc	*Gdnf*	heroin	↓	↑↑	(Airavaara *et al.*, 2011)
NAc	GDNF	heroin	–	–	(Airavaara *et al.*, 2011)
NAc	MeCP2, BDNF, TrkB (WB, qPCR)	heroin	–	–	(Theberge *et al.*, 2012)
NAc	*Oprm1*	heroin	↓	–	(Theberge *et al.*, 2012)
core	DAT (autoradiography)	cocaine	n.d.	–	(Ben-Shahar *et al.*, 2006)
core	D1, D2, NMDA (autoradiography)	cocaine	–	–	(Ben-Shahar *et al.*, 2007)
core	mGluR1, NR2A, NR2B	cocaine	–	–	(Ben-Shahar *et al.*, 2009)
core	mGluR5, Homer2a/b	cocaine	–	↓*	(Ben-Shahar *et al.*, 2009)
core	Homer1b/c	cocaine	↓*	–	(Ben-Shahar *et al.*, 2009)
core	CP-AMPAR activity	cocaine	–	↑	(Conrad *et al.*, 2008)
core	D1	cocaine	–	–	(Conrad *et al.*, 2010)
core	D2	cocaine	–	↓	(Conrad *et al.*, 2010)
core	D3	cocaine	–	↑	(Conrad *et al.*, 2010)
core	GLT1	cocaine	↓*	↓↓*	(Fischer-Smith *et al.*, 2012)
core	bCBV	cocaine	n.d.	↓	(Gozzi *et al.*, 2011)
core	rCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
core	BDNF	cocaine	–	↑	(Li *et al.*, 2013b)
core	*Htr6*, *Htr1b* (ISH)	cocaine	–	–	(Neumaier *et al.*, 2009)
core	AMPA (binding)	1d-cocaine	↓	–	(Halbout *et al.*, 2014)
core	NMDA (binding); *Grm1*	1d-cocaine	–	–	(Halbout *et al.*, 2014)
core	CP-AMPAR activity	meth	–	↑	(Scheyer *et al.*, 2016)
core	ratio AMPA/NMDA, CP-AMPAR	chow food	–	↑	(Dingess *et al.*, 2017)
core	ratio AMPA/NMDA, CI-AMPAR	fat food	–	↑	(Dingess *et al.*, 2017)
core	ratio AMPA/NMDA	sucrose	–	↓	(Counotte *et al.*, 2014)
core	DARPP-34 pThr75	nicotine	–	↓	(Abdolahi *et al.*, 2010)
core	DARPP-34 total, pThr34	nicotine	–	–	(Abdolahi *et al.*, 2010)
core	$GABA_A\alpha2$	cocaine	↑	–	(Purgianto *et al.*, 2016)
core	PV, calretinin, calbindin, nNOS	cocaine	n.d.	–	(Purgianto *et al.*, 2017)
core	$GABA_A\alpha1/4$	cocaine	–	–	(Purgianto *et al.*, 2016)
core<PL	synaptic strength	cocaine	↑	↑	(Luís *et al.*, 2017)
core<PL	silent synapses	cocaine	↑	–	(Ma *et al.*, 2014)
core<PL	CI-AMPAR activity	cocaine	n.d.	↑	(Ma *et al.*, 2014)
core<PL	synaptic strength (I/O curve)	cocaine	n.d.	↓20Hz	(Purgianto *et al.*, 2017)
core<BLA	synaptic strength (I/O curve)	cocaine	n.d.	–	(Purgianto *et al.*, 2017)
shell	DAT (autoradiography)	cocaine	n.d.	–	(Ben-Shahar *et al.*, 2006)
shell	D1 (autoradiography)	cocaine	↑	–	(Ben-Shahar *et al.*, 2007)
shell	D2, NMDA (autoradiography)	cocaine	–	–	(Ben-Shahar *et al.*, 2007)
shell	mGluR1	cocaine	–	↑*	(Ben-Shahar *et al.*, 2009)
shell	mGluR5, Homer1, Homer2, NR2A, NR2B	cocaine	–	–	(Ben-Shahar *et al.*, 2009)
shell	D1	cocaine	↑	–	(Conrad *et al.*, 2010)
shell	D2	cocaine	↓	↓	(Conrad *et al.*, 2010)
shell	D3	cocaine	–	–	(Conrad *et al.*, 2010)
shell	GLT1	cocaine	↓	↓*	(Fischer-Smith *et al.*, 2012)
shell	bCBV, rCBV	cocaine	n.d.	–	(Gozzi *et al.*, 2011)
shell	BDNF	cocaine	–	↑(wd90)	(Li *et al.*, 2013b)
shell	*Htr6*, *Htr1b* (ISH)	cocaine	–	–	(Neumaier *et al.*, 2009)
shell	AMPA (binding)	1d-cocaine	↓	↑	(Halbout *et al.*, 2014)
shell	NMDA (binding)	1d-cocaine	↓	–	(Halbout *et al.*, 2014)
shell	*Grm1*	1d-cocaine	↓	↓	(Halbout *et al.*, 2014)
shell	DARPP-34 total, pThr34, pThr75	nicotine	–	–	(Abdolahi *et al.*, 2010)
shell<IL	silent synapses	cocaine	↑	–	(Ma *et al.*, 2014)
shell<IL	CP-AMPAR activity	cocaine	n.d.	↑	(Ma *et al.*, 2014)
shell<BA	silent synapses	cocaine	↑	–	(Lee *et al.*, 2013)
shell<BA	CP-AMPAR activity	cocaine	n.d.	↑	(Lee *et al.*, 2013)

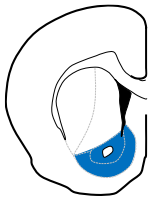

NAc


core


shell

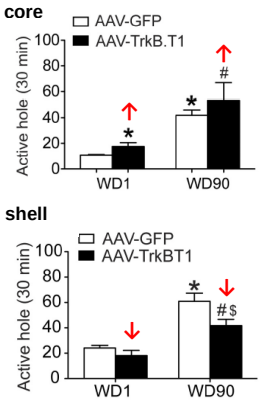
Involvement of brain regions in the incubation of seeking

a) Nucleus accumbens

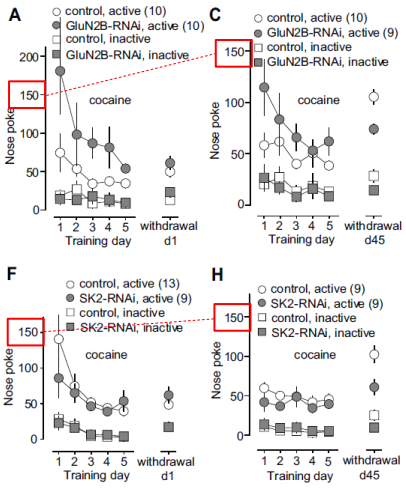


The nucleus accumbens, both its core and shell regions, is by far the region that has received most attention in the study of the incubation of seeking. However, only eight studies are based on manipulations performed at short and long abstinence times. Specifically, one with heroin (without distinction between regions), one with sucrose (distinguishing between core and shell regions) and six with cocaine (one in NAc in general, two in core, two in shell and one in both regions).

In the heroin study (Airavaara *et al*., 2011), the authors studied the effect of intraaccumbens administration of GDNF just after the last self-administration session. They observed that, although the treatment increased the seeking during the early extinction test, it did not exert any effect on the delayed test. In the study with sucrose (Grimm *et al*., 2011) observed that the administration of a dopamine receptor antagonist D1 (SCH23390), either in the core or shell, caused a decrease in the seeking of rats at short and long periods of abstinence, as well as a general locomotor depletion. However, the same systemic treatment caused differential decreases in seeking at short or long times of abstinence. In the cocaine studies it was observed that the BDNF-TrkB pathway is overexpressed after long periods of abstinence (45 days) in the core, and after 90 days in the shell (Li *et al*., 2013b). In this article, the authors studied the effect of attenuating that pathway using viral vectors expressed in core or in shell before self-administration sessions. They found that this treatment had opposite effects depending on the affected region, consistent with the antagonistic function that these regions seem to have on cocaine seeking: when it was in core, the effect was an enhancement of the seeking response, and when it was in shell the effect was the opposite. However, they did not see any effect on incubation, despite the fact that the authors discuss their results as if an effect had been found. At first sight it is observed that the incubation is not affected, since the groups have parallel increases in the seeking throughout the abstinence. But although they do not perform the appropriate factorial ANOVA and only compare the groups by independent Student *t*-tests, they discuss the differences and the lack of them as if they were effects of an interaction (and therefore an incubation effect) and not mere effects of the treatments.



Li *et al*., 2013b



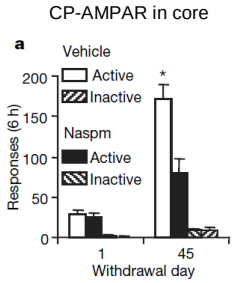
Wang *et al*., 2018

The application of an iRNA against the *Grin2b* transcript, in the shell (a transcript that is increased after short abstinence), blunted the incubation phenomenon. The same effect had the silencing of the Sk2 channels. However, if we analyze the self-administration sessions, the animals that were used for long-term abstinence consumed half the amount of drug than those dedicated to short times (Wang *et al*., 2018). This, by itself, can be the reason why these animals do not present incubation, since the phenomenon is related to the degree of exposure to the substance. However, the authors of the article do not further analyze this possibility or comment on it. In a study of the same year they observed that the silencing of the *Dnmt3a2* gene in shell caused a decrease in the responses, both after short times and long times of abstinence (Cannella *et al*., 2018). Given that the shell region is involved in the inhibition of inappropriate behaviors (Everitt and Robbins, 2016), this manipulation could be facilitating its function during the extinction test. Other researchers have already discovered that the inhibition of Dnmt enzymes causes a decrease in cocaine seeking, as discussed in the Cannella study.

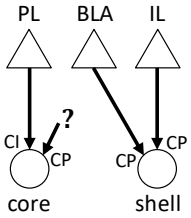
Dikshtein *et al*. (2013) observed that the endogenous opioid β-endorphin increased in nucleus accumbens during the extinction test at short abstinence times, but not at long times. The source of β-endorphin in the nucleus accumbens is the arcuate nucleus of the hypothalamus, related to appetite and satiety, and controlled, in part, by the central and medial nuclei of the amygdala (Zséli *et al*., 2018). To study their possible involvement in the incubation phenomenon, they neutralized this peptide with antibodies at short times, causing an increase in the seeking. At long times they injected the peptide in the same region, causing a decrease in the seeking. This effect was mediated by delta opioid receptors, based on the results with specific inhibitors of the delta (naltrindole) and mu (CTAP) receptors.

In addition, the administration of naltrindole alone to short periods of abstinence also caused an increase in seeking, an effect that was not observed at long times. The same treatment with CTAP did not produce effects on the seeking.

In another study by the group of Marina Wolf they observed that the administration in core of an inhibitor of CP-AMPA receptors (Naspm), that are increased in core after the delayed abstinence (and to a lesser extent also in shell), reduced the seeking to a dose that does not have this effect after a short abstinence (Conrad *et al*., 2008). The same effect of Naspm has been observed, although only studied at long periods of abstinence, after the incubation of methamphetamine seeking (Scheyer *et al*., 2016). Related to changes in CP-AMPAR, the administration of a positive allosteric modulator of mGluR1 receptors in the core also prevented the incubation of cocaine seeking, although, again, it was only only studied at long abstinence times (Loweth *et al*., 2014a). Overexpression of the Homer subunits in the core region had no effect on incubation (Loweth *et al*., 2014b). The effects of AMPA receptors in the nucleus accumbens have been studied in considerable detail, but the origin of these CP-AMPAR-based synapses is unknown. It is known that if CI-AMPA receptors from prelimbic cortex (PL), in principle not involved in incubation, are resilenced in core, the seeking decreases (Ma *et al*., 2014); if the CP-AMPA receptors are resilenced in synapses from the basal region of the amygdala to shell, located in MSN-D2 neurons (Terrier *et al*., 2016), the seeking decreases (Lee *et al*., 2013); if CP-AMPA receptors are resilenced in shell at synapses from the infralimbic cortex (IL), located in MSN-D1 (Terrier *et al*., 2016), the seeking increases; and activating with optogenetic techniques the IL>shell path decreases the seeking (Müller Ewald *et al*., 2018). All these manipulations were performed exclusively after long periods of abstinence, so their involvement in the incubation phenomenon is not clear. In addition, contradictory results have been obtained using other substances. As when cocaine is used (Conrad *et al*., 2008), increases in the AMPA/NMDA ratio are seen during the incubation of food due to the insertion of CP-AMPAR, but of CI-AMPAR when a high-fat diet is used (Dingess *et al*., 2017). In addition, when the consumed substance is sucrose, decreases in the core are observed in this ratio (Counotte *et al*., 2014).



Conrad *et al*., 2008



Conrad *et al*., 2008
Lee *et al*., 2013
Ma *et al*., 2014

We observe the same gap (manipulations performed just at a single time point) when studying the effect of a D3 inhibitor in the shell or the core (Xi *et al*., 2013), only studied at long periods of cocaine abstinence; or rolipram (Sun *et al*., 2015), only studied at long heroin times. In both cases the seeking decreases.

In electrophysiology studies in which the animals are exposed to the cues associated with the substances and the neuronal activity is recorded, different results have been obtained again depending on to whether cocaine or sucrose seeking incubation was studied. While the incubation of cocaine provokes a greater activation of the core region by the cues (Hollander and Carelli, 2007), this is not the case with the incubation of sucrose seeking (Jones *et al*., 2008).

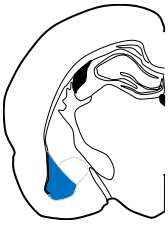
In studies in which animals were subjected to extinction tests and then sacrificed to measure activity by levels of Fos, pERK or pCREB at short and long times of abstinence, a greater Fos labeling in the shell and core in rats that had been administered sucrose (Grimm *et al*., 2016) and nicotine (Funk *et al*., 2016) was observed at long abstinence times, but lower labeling of pERK and pCREB in nucleus accumbens in rats that had been administered heroin (Sun *et al*., 2015). The same design has not been done with psychostimulants, but Gueye *et al*. (2018) found increases of BDNF in core only after protracted extinction test.

Therefore, and summarizing, the link of the nucleus accumbens with the phenomenon of the incubation of the seeking is not clear. Despite the high number of studies, the only one that performs the same manipulation at short and long periods of abstinence that is also effective only at long times is that of Conrad *et al*. (2008), but this manipulation has not been reproduced to date replacing cocaine with another substance.

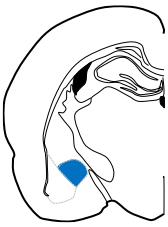
b) Amygdala

Another frequently studied region is the amygdala, generally differentiating between the basolateral (BLA) and central (CeA) regions. Also here, only a few

studies are based on manipulations at both short and long abstinence times. Specifically three: one in which the authors study the two regions mentioned above (focusing on methamphetamine), and two in which they study only the central nucleus of the amygdala (one with cocaine and the other with sucrose).



In the study with methamphetamine, the authors inactivated CeA or the basal amygdala (BA) region of BLA with the GABAergic muscimol/baclofen agonist cocktail before the extinction test and compared relapse with vehicle-treated rats. The authors observed that only manipulation in CeA caused declines in response and only after long periods of abstinence (Li *et al*., 2015b). In the other two studies the authors used the same manipulation but only in CeA. They inactivated with LY379268, agonist of glutamatergic autoreceptors mGluR2/3, and observed that relapse decreased only after long periods of cocaine abstinence (Lu *et al*., 2007; do not observed this effect in BA, although they only studied this region after long periods of abstinence) and sucrose (Uejima *et al*., 2007).



In addition to these, there are six other studies that only look at the effects at short times or long periods of abstinence. With the exception of the aforementioned study by Lee *et al*. (2013) in which the authors manipulated the BA>shell path, it was the manipulation in CeA and not in BLA that had effects at protracted abstinence. There are two studies with cocaine (Lu *et al*., 2005b, in BA; Xi *et al*., 2013), one with methamphetamine (Venniro *et al*., 2017a), one with nicotine (Funk *et al*., 2016) and one with a CPP protocol using morphine (Li *et al*., 2008). In the work of Venniro *et al*. (2017a), the authors observed that the inhibition of the pathway from the ventral anterior insular cortex to CeA decreased relapse. In addition, they observed that the inhibition of D1 receptors with SCH39166 had the same effect, but not the inhibition of the D2 receptors with raclopride. The release of dopamine in CeA is necessary for the attentional processes, and it seems that the D1 receptors, and not the D2 receptors, play a prominent role (Smith *et al*., 2015).

Studies have also been carried out in which the rats are subjected to extinction tests and then sacrificed to study the activation of different regions by quantifying pERK, Fos or equivalents. In all studies, greater activation of CeA was observed at long abstinence times, whether using cocaine (Lu *et al*., 2005b; Thiel *et al*., 2012), morphine in CPP paradigms (Li *et al*., 2008) , sucrose (Grimm *et al*., 2016) or nicotine (Funk *et al*., 2016). The activation of CeA was prevented with an environmental enrichment treatment that lowered cocaine seeking levels (Thiel *et al*., 2012). Of these four studies, which also quantified the activation of BLA and are the only ones in which the authors also studied the effect at short times, only in that of Funk *et al*. (2016) an activation of the BLA region was observed.

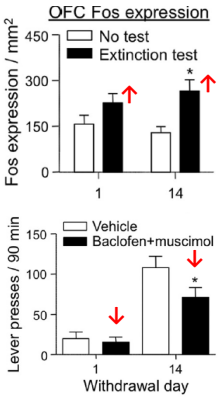
There are three other studies in which the authors only quantified the activation after protracted abstinence: two with methamphetamine and one with alcohol, substances with which an equivalent study has not been published looking at both times. In the study by Li *et al*. (2018), the authors observed Fos increases in BLA after methamphetamine seeking incubation, but not in Venniro *et al*. (2017a), in which they did observe CeA activation. In the study by Radwanska *et al*. (2008) with alcohol, the authors observed activation of the subregion CeC/L but not of CeM or MePD divisions of the central amugdala. In the basolateral region, they observed an activation of the BA, lateral ventral and BMP regions, but not lateral dorsal .

In short, unlike the basolateral region, the central nucleus of the amygdala does seem to have a common role in the incubation of seeking.

c) Prefrontal cortex

Although there are thirteen articles that study the possible involvement of the prefrontal cortex (PFC) in the phenomenon of incubation through pharmacological manipulations, only five of them perform them both at short and at long times of abstinence. Four of them studied the possible involvement of medial PFC (mPFC) with psychostimulants (cocaine and methamphetamine) and the fifth study focuses on the possible involvement of the orbitofrontal cortex (OFC) in the incubation of heroin seeking.

In the OFC study (focusing on heroin seeking incubation), Fanous *et al*. (2012) found that extinction test differentially induced changes in Fos reactivity in lOFC. Then, inactivation of the lateral region (lOFC) with muscimol/baclofen inhibited relapse after long periods of abstinence but not after short times. However, as the authors describe in the text, they observed neither an effect of the cocktail of inhibitors nor of the interaction between this treatment and the duration of abstinence. The differential effect is justified based on comparisons between groups by uncorrected multiple comparisons. By inactivating only those neurons that are activated with the associated cues, they observed a decrease in relapse. However, they tested this manipulation only after protracted abstinence. This same manipulation and also only after a long period of abstinence was carried out in Funk *et al*. (2016) with nicotine, but without any effect. Li *et al*. (2015b) or Venniro *et al*. (2017a) inhibited the lOFC with muscimol/baclofen, also only at long abstinence times, finding no effects on methamphetamine seeking incubation. In studies with extinction test and subsequent measurement of Fos, Fanous *et al*. (2012) observed an increase in this region, but again it seems more due to an increase at both times of abstinence than a differential increase after long periods. As a results, the authors chose to perform the same (inadequate) statistical analysis as before. Funk *et al*. (2016), studying nicotine seeking incubation saw differential increases after long abstinence, but not Grimm *et al*. (2016) after incubation of sucrose seeking.



Fanous *et al*., 2012

Dysfunctions can also be inferred in certain regions of the brain through tests whose neurophysiological basis is known. Reversal learning, for example, seems to depend on the OFC and its interaction with the amygdala (Izquierdo *et al*., 2017). In a study on incubation of cocaine seeking, Calu *et al*. (2007) observed an impairment of this type of learning. This could be related to the decrease in rCBV (only studied at long abstinence times) in OFC published by Gozzi *et al*. (2011). However, also studying cocaine, Gobin and Schwendt (2017) did not find any change in reversal learning, although they did in the working memory, also dependent on the PFC. However, data did not correlated with the seeking of the animals. Both studies performed the tests only at long abstinence time.

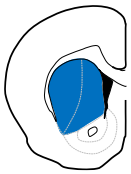
Swinford-Jackson *et al*. (2016) observed a decrease in the 5HT2C serotonin receptors in mPFC after incubation of cocaine seeking but not sucrose. Subsequently, and only with cocaine, the authors tested whether the activation of these receptors with WAY163909 affected differently to short and long abstinence times. Indeed, they found that at longer times this intervention had lower capacity to decrease seeking. In an incubation study using CPP with KO heterozygous mice for the extracellular matrix protein brevican, Lubbers *et al*. (2016) observed that overexpression of this protein in the dorsal region of mPFC (dmPFC) did not affect incubation. In a study with self-administration of cocaine, Gould *et al*. (2015) observed changes in different isoforms of Homer proteins in the ventral region of mPFC (vmPFC). The reversal of such changes, however, did not affect relapse. Finally, the inactivation of the infralimbic region by muscimol/baclofen also did not affect the incubation of methamphetamine seeking (Li *et al*., 2015b).

In pharmacological studies in which the same treatment is only performed at short or long abstinence time, were observed different results depending of the manipulated region (dmPFC, vmPFC). Specifically, a treatment with muscimol/baclofen in dmPFC did not cause changes in relapse to cocaine (Koya *et al*., 2009) or methamphetamine (dPL; Li *et al*., 2015b). By inhibiting PI3K kinase in PL, the cocaine seeking is reduced (Szumlinski *et al*., 2018). This region contains a dorsal part (dPL) belonging to dmPFC and a ventral part (vPL) belonging to vmPFC. Three different treatments in vmPFC reduced the relapse to cocaine: with muscimol/baclofen (Koya *et al*., 2009), activating the mGluR1/5 receptors with DHPG (Ben-Shahar *et al*., 2013, although only after repeating the extinction test), and inhibiting PKCε (Miller *et al*., 2017). However, since these treatments were only performed at a single time, it is impossible to know if the reported effects were due to a differential action after incubation or a general effect on the seeking, regardless of the time of abstinence. However, Müller Ewald *et al*. (2018) found that the optogenetic activation of IL only affected the seeking if the animals had previously gone through extinction sessions, unlikely if the IL>shell pathway was specifically activated. This could explain the result of the only study testing an activation, and not an inhibition, of the region (Ben-Shahar *et al*., 2013).

There are several studies in which the animal is exposed to an extinction test and then a differential change is observed. Specifically, seven with cocaine, two with sucrose and one with nicotine. In studies with cocaine in which they measured parameters directly related to the activity, they found apparently contradictory results. While Koya *et al*. (2009) observed increases in pERK in both regions, West *et al*. (2014) observed increases in reactive neurons only in PL, but not in IL, and Gueye *et al*. (2018) found increases of BDNF in PL. This could be due to the fact that the region causing the increases in vmPFC in the first article was the ventral part of PL (vPL), located in both vmPFC and PL. Studying sucrose seeking incubation, Grimm *et al*. (2016) observed increases in Fos in PL, ACC and IL, and studying nicotine, Funk *et al*. (2016) reported Fos increases in dmPFC and vmPFC. Ben-Shahar *et al*. (2013) and Miller *et al*. (2017) observed changes in vmPFC that did not occur in dmPFC: the former found decreases in mGluR1/5 level, and the later observed increases in PKCε phosphorylation. Finally, and comparing the effect of an extinction test on the incubation of cocaine and sucrose, Shin *et al*. (2016) observed dopamine decreases and glutamate increases in vmPFC in cocaine experiments, but none of these changes were observed for sucrose.

It seems, therefore, that both regions, dmPFC and vmPFC, could have a role in the incubation phenomenon, but maybe not in the same way with the different substances. As there are only a few studies of pharmacological manipulation at both times of abstinence dealing with cocaine seeking incubation, the degree of involvement of the different regions in the phenomenon is not clear.

d) Dorsal striatum



There are four articles in which the same manipulation is performed in regions of the dorsal striatum at both times of abstinence: one using cocaine and three using methamphetamine. Therefore, the dorsal striatum has only been studied in this way with psychostimulants.

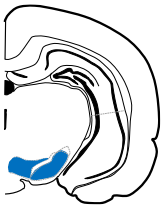
Although there are just a few studies, the most plausible conclusion is that the dorsomedial region, and not the dorsolateral region, is involved in the expression of the seeking incubation. If dopamine D1 receptors are inactivated in the central region (comprised of a lateral and a medial part), a differential affectation of the seeking after protracted abstinence is observed (Li *et al*., 2015d). This may be due to its lateral part, its medial part, or some kind of functional specificity of the central region. When the dorsolateral region is inactivated with GABAergic agonists (muscimol/baclofen) after cocaine consumption, the same decrease in seeking to both withdrawal times is observed (Pacchioni *et al*., 2011). On the other hand, if dopamine D1 or D2 receptors are inhibited in the medial region after methamphetamine consumption, seeking is differentially affected (Caprioli *et al*., 2017a), and the same happens inactivating the projection to DMS from the lateral intralaminar nucleus of the thalamus (AIT-L>DMS), with simultaneous injections, ipsi or contralaterally, of a D1 inhibitor in the striatum and muscimol/baclofen in the thalamus (Li *et al*., 2018).

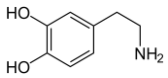
In spite of this, only one study looks for differential effects of the extinction test at both times of abstinence in the dorsomedial striatum, while in four publications they try to find these effects in the dorsolateral striatum (DLS), a region classically involved in addictive processes.

Werner *et al*. (2015) found differential changes in protein degradation pathways after cocaine consumption and abstinence in DLS. Grimm *et al*. (2016) observed greater presence of Fos after prolonged periods of abstinence in DLS. Li *et al*. (2015d) studied differential changes in gene expression after abstinence from methamphetamine in DLS. Finally, Caprioli *et al*. (2017b), studying Fos in DLS and DMS, observed a differential increase after long abstinence times only in DMS, in both D1 and D2-MSN.

It seems therefore that seeking incubation is not so much an incubated habit but rather a goal-directed behaviour, given the involvement of the dorsomedial striatum in contrast to the obtained results in the dorsolateral striatum. Or perhaps it is the sum of both types, and the function of the dorsal striatum is merely to allow the expression of the phenomenon.

e) Dopaminergic system





There are only two published studies performing manipulations in dopaminergic regions at both withdrawal times, one studying cocaine and another one focused on heroin. The authors studied the effects of acute and chronic treatments (tmt) of GDNF both in the ventral tegmental area (VTA) and in the substantia nigra pars compacta (SNpc): injection of GDNF just after the last self-administration session (tmt1), inhibition of ERK phosphorylation (effector of the GDNF receptor) just after the last self-administration session (tmt2), injection of the *Gdnf* mRNA during withdrawal (tmt3), and injection of an antibody to GDNF throughout abstinence (tmt4). Lu *et al*. (2009) studied tmt1-4 manipulations in VTA and tmt1 in SNpc after cocaine use. Airavaara *et al*. (2011) studied tmt1 and tmt4 (this later only after protracted abstinence) in VTA after the heroin consumption. The only treatments that produced a differential effect between both times of abstinence were tmt3 (increases) and tmt4 (decreases) and only after the consumption of cocaine. This effect, in addition to being specific for cocaine, may simply be a cumulative effect, since the late test occurred after more treatment doses than the early test.

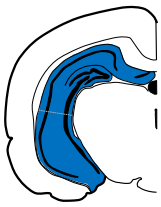
In studies of the effect of the extinction test on immediate early genes (Fos), Grimm *et al*. (2016) did not find differential changes in either of the two regions after sucrose consumption. Gueye *et al*. (2018) found increases of BDNF in VTA only after cocaine protracted extinction test. By quantifying the release of dopamine in vmPFC during the test, the authors of another study found a decrease in this catecholamine after the incubation of cocaine seeking but not after sucrose (Shin *et al*., 2016).

When instead of manipulations in the dopaminergic nuclei antagonists of dopamine function were injected, we generally observe that the seeking descends. The problem, again, is that the same manipulation is almost never done at both times of abstinence. An *i.p*. administration of SCH39166, inhibitor of D1 receptor, decreased the seeking of sucrose at both times (Grimm *et al*., 2011), and that of methamphetamine at least at long times (Venniro *et al*., 2017a). The administration of SB-277011A, inhibitor of D3 receptors, did the same with cocaine seeking, at both times (Xi *et al*., 2013). On the other hand, the systemic administrations of D1 or D2 agonists did not cause changes in the seeking of sucrose at any time (Glueck *et al*., 2017). When they were administered in brain regions, the picture is somewhat different. Considering the articles in which treatments were performed at both times of abstinence, the inhibition of D1 in DMS, as well as that of D2, decreased the seeking of methamphetamine after long periods (Caprioli *et al*., 2017b), and the inhibition of D1 in the core, as well as in the shell, equally affected the seeking of sucrose at both times of abstinence (Grimm *et al*., 2011). Related to this last study, the inhibition, although only studied at long times of abstinence, of D3 in those same regions, decreased cocaine seeking (Xi *et al*., 2013).

In the amygdala, although only studied at long abstinence times, the inhibition of D1 caused decreases in methamphetamine seeking when performed in CeA (although not with D2 inhibitors) but not in BLA (Venniro *et al*., 2017a). Likewise, the inhibition of D3 in CeA but not in BLA caused decreases in cocaine seeking (Xi *et al*., 2013). In summing up, we could say that if dopamine were to have a single function in the incubation of the seeking of any substance, this could be to allow its expression. There does not seem to be a differential function in BLA or nucleus accumbens, neither the core nor the shell, although it could be in CeA (D1 but not D2 receptors) and DS (D1 and/or D2 receptors). This conclusion is interesting because CeA is able to modulate SNpc, which in turn releases dopamine in DS.

Therefore, the implication of these two dopaminergic regions in the phenomenon of incubation of seeking is not clear, although it seems that they are not directly involved in it. However, dopaminergic function seems necessary for the incubation of the seeking to be expressed.

f) Hippocampus



There are two articles which study the possible involvement of the hippocampus in the incubation of cocaine seeking. In one of them (Karlsson *et al*., 2013) the authors do not observe any effect when lesioning the ventral region of the hippocampus (vHPC). On the other hand, in a protocol of CPP with cocaine (Lubbers *et al*., 2016), heterozygous mice KO for brevican did not show incubation, but when the protein was induced in the dorsal region of the hippocampus (dHPC) incubation was restored. After the protracted withdrawal of sucrose (Grimm *et al*., 2016) a differential effect of the extinction test on Fos was not observed neither in dHPC nor in the ventral subiculum (vSub).

It seems therefore that the involvement of the hippocampus in the incubation of the seeking might depend more on the paradigm used (CPP versus self-administration), although there are no studies manipulating dHPC in self-administration protocols.

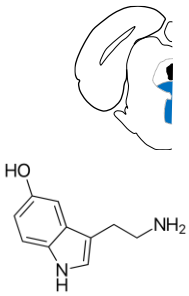
g) Hypothalamus and peptidergic systems

The hypothalamus and peptidergic systems (such as opioids, CRH or orexin) have not been widely studied in relation to the incubation of seeking, however, some studies are available. The issue here is that there is no overlap between the parameters analysed in articles that use cocaine and those that use opiates (heroin, oxycodone).

After two weeks of abstinence after cocaine consumption the basal and reactive activity (bCBV and rCBV) of HPT are unchanged (Gozzi *et al*., 2011). However, there is an early increase of orexin$^+$ neurons in the DMH/PF and LH regions that returns to basal levels after delayed abstinence in DMH/PF but not in LH, without changes in the number of MCH$^+$ neurons in any of the regions (James *et al*., 2018). In parallel, Steiner *et al*. (2018) observed that Hcrt KO mice incubated cocaine seeking, although the effect disappeared earlier than in WT mice. The arcuate nucleus is responsible for the secretion of β-endorphin in NAc. Dikshtein *et al*. (2013) saw increases in this opioid in NAc during the early cocaine abstinence test (without long-term effects). Blocking it with specific antibodies led to increases in the seeking. Theberge *et al*. (2012), using heroin, found decreases in *Oprm1* in that region (no changes in DS), also only during early abstinence. Since the effects appear to be due to their action on the DOR but not MOR receptors (according to the results with specific antagonists), this reduction of the MORs after heroin consumption could be a homeostatic change. Another possibility is that both results are specific to each of the substances.

Blackwood *et al*. (2018) studied the changes in gene and protein expression of mu, delta and kappa receptors after protracted oxycodone abstinence in DS and HPC. The authors observed decreases of MOR in DS and its mRNA, *Oprm1*, in HPC. Shalev *et al*. (2001), on the other hand, did not observe changes in *Crh* at any time neither in CeA nor in BNST (dorsal or ventral). Finally, Freeman *et al*. (2008), studying the gene expression of CART and NPY neuropeptides in mPFC and NAc, observed increases of both in mPFC at short times of abstinence.

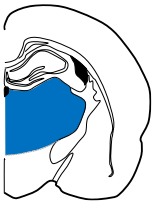
h) Serotoninergic system



As in the previous sections, almost all studies dealing with the serotoninergic system have focused on the incubation of cocaine seeking. Gozzi *et al*. (2011) found that, at least after two weeks of forced abstinence from cocaine, the baseline activity (bCBV, but not rCBV) of the raphe nuclei was below what was expected. Measured at both times of abstinence, they also found decreases in the expression of serotonin receptors in DMS, NAc and mPFC. In DMS, decreases in the *Htr1b* autoreceptor were evident at long abstinence times, but no changes in *Htr6* at any time or changes in either of them in the NAc (Neumaier *et al*., 2009), a region in which *Htr1d* autoreceptor decreases were found after short periods of abstinence (Freeman *et al*., 2010). Decreases in autoreceptor levels could cause increased

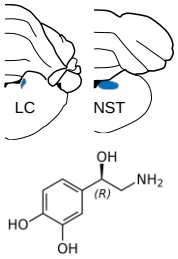
effects of serotonin in these regions. In mPFC, lower levels of 5HTR2C were found after long periods of abstinence in rats that consumed cocaine but not in those that consumed sucrose. Activation of them had a less inhibitory effect on cocaine seeking after protracted abstinence (Swinford-Jackson *et al*., 2016). This receptor, with upwards neuromodulatory capacity, seems to be located in interneurons in mPFC, and its activation causes decreases in seeking (Pentkowski *et al*., 2010). Therefore, a depletion of this receptor could translate into a lower capacity to control the seeking by this region in circumstances in which the level of 5HT increases. In the study by Hamed and Boguszewski (2018) on the incubation of CPP with morphine, the extinction test after long periods of abstinence caused increases in total 5-HT in mPFC, Amg, HPC and DS (but not in NAc or VTA).

i) Thalamus



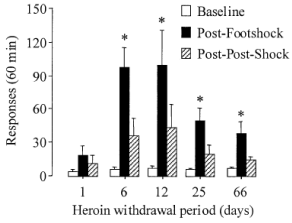
The involvement of the thalamus in the incubation phenomenon has only been examined in two articles, both of them focusing on a psychostimulant. Gozzi *et al*. (2011) observed decreases in rCBV (but not bCBV) in that region at long cocaine withdrawal times (they did not study early abstinence). On the other hand, using methamphetamine, Li *et al*. (2018) were interested in the region of the thalamus that projects to the DMS and that was activated during the extinction test after long periods of abstinence (shorter periods were not included in the study). They noted that it was AIT-L. Its bilateral, but not unilateral, inactivation reduced the seeking at such time. More interestingly, both ipsi and contralateral disconnection of the AIT-L>DMS pathway (with muscimol/baclofen in AIT-L and a D1 inhibitor in DMS) elicited the same effect at long abstinence time but not at short times. Therefore, it seems that the AIT-L>DMS pathway is important for the incubation of methamphetamine seeking expression.

j) Noradrenergic system



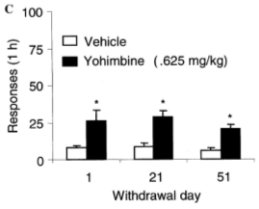
The main noradrenergic nuclei sending projections to limbic regions are the locus coeruleus (LC), which sends its projections through the dorsal bundle to the glutamatergic regions PFC and BLA, and the nucleus of the solitary tract (NST), which sends its projections through the ventral bundle to the GABAergic regions shell and CeA (Kvetnansky *et al*., 2009). The only article related to the noradrenergic system known to us is that of Hamed and Boguszewski (2018) using a CPP protocol with morphine. They measured the total level of noradrenaline and its main metabolite, MHPG, after the extinction test, in NAc, DS, mPFC, HPC, Amg and VTA. They observed decreases of the metabolite in NAc after a long period of abstinence.

Effects of stress and environmental enrichment. Various studies have attempted to understand the relationship between stress (in its broadest sense) and the incubation of seeking. For example, it has been studied if the stress-induced relapse incubates, if the resistance to punishment incubates, if the incubation of cue-induced seeking is affected by the level of stress or if molecules associated with it are involved in the phenomenon. Likewise, it has been studied how environmental enrichment affects incubation of cue-induced seeking.



Shalev *et al*., 2001

One of the causes of relapse is stress, consequently, it is important to determine if stress-induced seeking incubates. Two works have asked such question, following different strategies.. In the study by Shalev *et al*. (2001), the authors observed that footshocks had a increasing capacity to induce relapse throughout abstinence both in rats that had consumed heroin and in those that had consumed sucrose (in this case the increases are not significant). In another study, injecting yohimbine ($\alpha 2$ adrenergic antagonist) into rats that had consumed methamphetamine, did not induce stress-induced seeking incubation (Shepard *et al*., 2004).

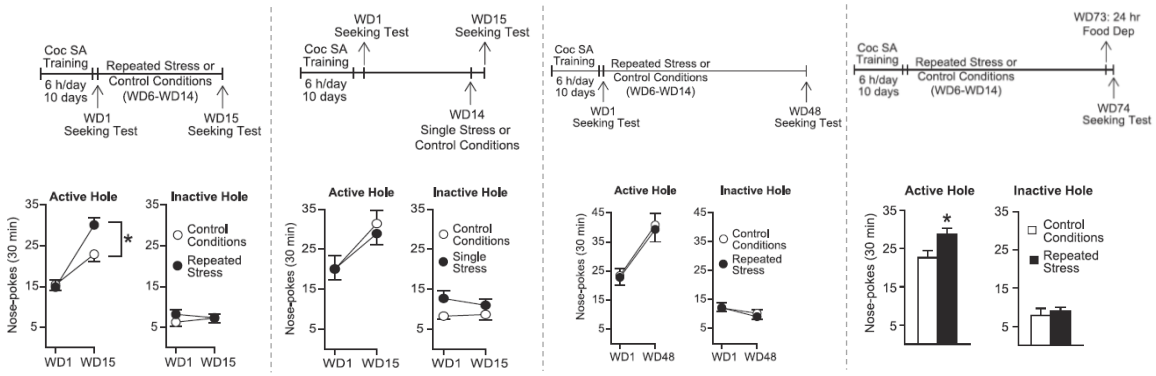


Shepard *et al*., 2004

It has also been studied, at least in one article, whether the *resistance to punishment* during consumption incubates or not. To attain this goal, they accompanied each infusion of cocaine with an aversive dose of histamine. They observed that rats that had more time of abstinence consumed more cocaine, hence the authors inferred that the rats had become more resistant to punishment (Gancarz-Kausch *et al*., 2014). However, it can also be interpreted as follows: rats, while being equally resistant (or sensitive) to punishment, have suffered an incubation of re-acquisition, something already proven by others with cocaine and other

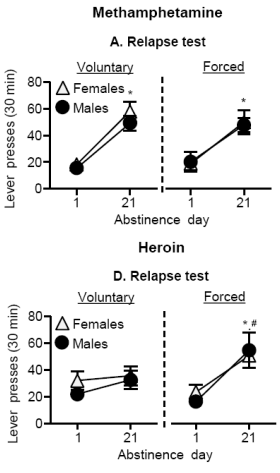
substances (see *Box 7. Paradigms in which incubation has been observed*). In a similar study, using lithium chloride as aversive in a conditioned taste aversion protocol (CTA) after sucrose consumption, the authors observed that CTA was only effective to reduce consumption if the unconditioned stimulus was applied after one month of abstinence (Harkness *et al*., 2010).

In addition to this, another focus of interest has been to determine to what extent stress affects the incubation of cue-induced seeking, as well as the effects of environmental enrichment. Caloric restriction can be considered as a stress for the organism. In addition, as discussed in D'Cunha *et al*. (2017), drug use and *caloric restriction* affect each other in drug addicts: a higher consumption of drugs is associated with a decrease in food intake, and a caloric restriction is associated with an increase in drug use. Using caloric restriction as a stressor, D'Cunha *et al*. (2013) observed that, if suffered chronically during abstinence, it had the capacity to increase the incubation of heroin seeking, an effect that was not given if the restriction was acute or if the day before the animals were food satiated. Using an *immobilization stress* protocol, Glynn *et al*. (2018) managed to increase the incubation of cocaine seeking as long as it was chronic and recent or, if not recent, as long as the animals were subjected to caloric restriction the day before the test. Interestingly, when the seeking was tested one month later, without stress, the levels returned to those of the control animals.



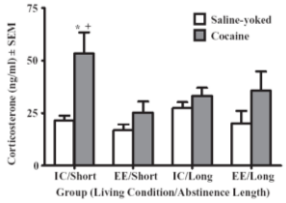
Glynn *et al*., 2018

The problem with these studies is that the treatment (stress) is applied only before subjecting the animals to the late extinction test and not to both abstinence times. Another strategy used is to induce *semi-voluntary abstinence* in animals by applying a footshock half of the animals seek the substance (lever press or nose poke). The animals end up abstaining, although there are individual differences (in the case of those rats that consumed methamphetamine, there were more resistant and more sensitive individuals). After abstinence, the animals are subjected to a relapse test after different times and it is observed that, while those that consumed methamphetamine continue to exhibit an incubation of seeking (higher in the resistant ones, maybe due to a greater consumption during the voluntary abstinence), those that consumed appetitive food do not (Krasnova *et al*., 2017, 2014; Torres *et al*., 2017).



Venniro *et al*., 2017b

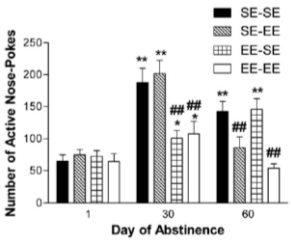
In contrast to caloric restriction, it has been shown that the contingent consumption of natural reinforcers (such as food) lowers stress levels (measured as plasma corticosterone concentration) (Ulrich-Lai and Herman, 2009). Using a different *voluntary abstinence* paradigm, in which animals stop consuming a drug to consume contingently a palatable food, it has been observed that methamphetamine seeking incubation is maintained (Caprioli *et al*., 2015b; Venniro *et al*., 2017b) but that of heroin disappears (Venniro *et al*., 2017b).



Thiel *et al*., 2012

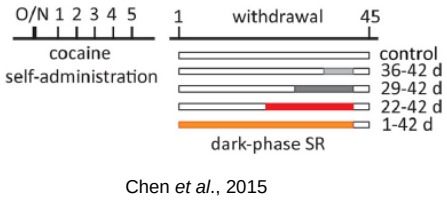
Two articles study the effect of the extinction test on *plasma corticosterone* levels: one after cocaine use (Thiel *et al*., 2012) and another after the consumption of sucrose (Grimm *et al*., 2016). Whereas after cocaine consumption an increase in the early abstinence test is observed, no changes are observed after the consumption of sucrose at any time of abstinence, although it is true that in this case the animals were anesthetized before sacrifice. In the cocaine study, they also observed that environmental enrichment, which reduced cocaine seeking, prevented the increase in plasma corticosterone.

Up to eight articles have studied the effects of *environmental enrichment* (EE) in the incubation of seeking, using cocaine and sucrose. The components of EE that have been used are aerobic exercise (a wheel), social interaction (more than one rat per cage) or novelty (an alternative cage, toys), in acute (one day) or chronic treatments (several days), recently or not (that is, with or without EE during the days before the test). However, the procedures have not been homogeneous in the different studies. For example, studies with cocaine always perform chronic EE (for several days) and therefore never study the effect on early abstinence, and in the studies with sucrose the EE is always recent (prior to the test) and therefore they do not study if the effects of EE are long lasting. The components with greater capacity to reduce the seeking after protocols that induce incubation seem to be aerobic exercise and toys (Zlebnik and Carroll, 2015: cocaine, EE = wheel, using only females; Grimm *et al*., 2008: sucrose, EE = wheel + social; Grimm *et al*., 2013: sucrose, EE = toys and/or social and/or novelty). An acute treatment (the day before) can achieve these effects (Grimm *et al*., 2016, 2013: sucrose), even better, without the need to prolong it over time for several days or weeks. It is not clear, however, what is the ultimate mechanism of the effect of environmental enrichment (Glueck *et al*., 2017; Grimm *et al*., 2016). The beneficial effect of EE seems not to be lasting, at least in rats that consumed cocaine, since its discontinuation, eventually leads to the disappearance of the effect (Chauvet *et al*., 2012: cocaine, EE = social + wheel + toys + novelty), unless an LTD is induced in the BLA>NAc path (Ma *et al*., 2016). The effect of this LTD on its own is also transient (Lee *et al*., 2013).

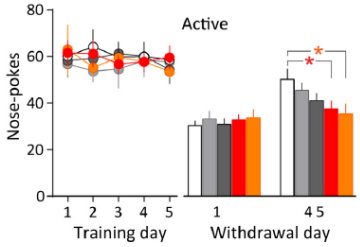


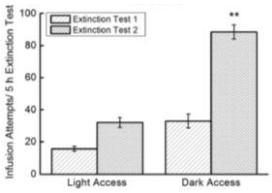
Chauvet *et al*., 2012

Although it is not directly related to stress, it has been seen that both the quality of *sleep* and whether drug consumption takes place in the active (dark) or inactive (illuminated) phase of the cycle affect the incubation of the seeking. As cocaine withdrawal progresses, the quality of sleep decreases. By improving it during withdrawal, the authors managed to decrease the seeking after prolonged abstinence (Chen *et al*., 2015). In addition, if rats had access to heroin during their natural phase of activity, that is, the dark phase, they consumed more and showed a greater incubation of the seeking (Coffey *et al*., 2018).



Chen *et al*., 2015



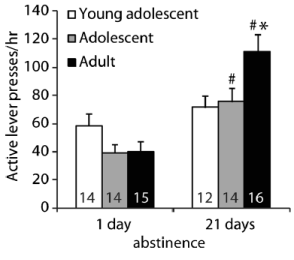


Coffey *et al*., 2018

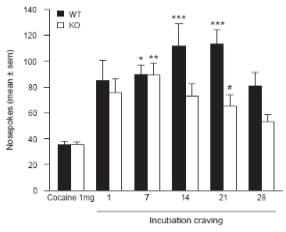
However, the level of stress of the animals during the extinction test affects the development of the same, but it is not clear to what extent the stress pathways and the molecules associated with it are involved with the incubation of the seeking.

Effects of sex. Incubation of seeking occurs in both male and female animals, at least when cocaine (Kerstetter *et al*., 2008), methamphetamine and heroin (Venniro *et al*., 2017b) were tested. However, the duration of the test is longer in female rats and its intensity even greater if they are in estrous on the day of the test (Kerstetter *et al*., 2008).

Effects of age. Up to four articles study the effect of age on seeking incubation. Using cocaine as the tested drug, Li and Frantz (2009) observed that, after a protocol of restricted access (2h/d), only adult rats (PND83-95) exhibited seeking incubation, and not the youngest ones (PND35). However, with an extended access protocol (6h/d) both groups (PND35 and PND70) exhibited an equivalent incubation in the seeking (Madsen *et al*., 2017). They also exhibited the same incubation in Funk *et al*. (2016) using nicotine and rats aged PND28-30 and PND68-70. However, after 10 sessions of 3h/d of self-administration of sucrose, it was observed again that the younger rats (PND35) did not present seeking incubation while the adults (PND70) did, with intermediate results in rats of PND42 (Counotte *et al*., 2014). Therefore, although it seems that a younger age does not prevent the potential for incubation of seeking, it is a factor of resistance.



Counotte *et al*., 2014



Steiner *et al*., 2018

Kinetics of seeking incubation. The phenomenon of incubation does not show the same time course in all the substances studied. This may be because they act on different processes or with different intensity, or because it is difficult to compare different substances with different dosages. For example, if each delivery of cocaine prolonged for 90 seconds instead of the typical fast delivery (around 5 seconds), the rats do not exhibit incubation (Gueye *et al*., 2018). In a general way, we could say that cocaine reaches the maximum in one month of abstinence, and heroin and methamphetamine in one or two weeks. While cocaine may last more than six months, the effect disappears in alcohol and heroin after two months (Figure 8, p.16) (Pickens *et al*., 2011). Keeping in mind that rats live between two and three years so it is not surprising that times are somewhat higher in people as we saw in the previous section. The only intervention that we have found that affects the kinetics of incubation is that published by Steiner *et al*. (2018). They note that in the Hcrt KO mice cocaine seeking incubation occurs as in the WT, but the effect ends earlier.

1.2.4. Summary of the state of the art in seeking incubation

Despite the huge number of studies that exist to date on the incubation of the seeking, it is difficult to point out a region of the brain or a mechanism that seems to be ultimately responsible for the process, regardless of the substance studied. This is not because equivalent results are not obtained among the substances but because there is hardly any overlap between the parameters that have been studied in some substances and those that have been studied in others. To make matters worse, it is not common to find studies of manipulation that study equally short times and long times of abstinence, and in those investigations that measure biochemical parameters it is rare to find a statistical analyses that allows to discern between the lasting effects of drug intake, the effects of the passage of time during the period of abstinence and the effects of the interaction between both factors, this last requirement indispensable to be able to suggest the potential existence of an incubation-related effect. It is important to take this into account when extracting the pertinent information without confounding it with mere effects of seeking or lasting changes derived from the specific intake of a given substance. For all this, our purpose has been, in this Introduction, to look for processes common to the incubation of the seeking of the various substances studied. Leaving aside the changes in biochemical parameters, which, apart from the problem of statistical analysis, are almost all focused on cocaine, common effects in the experiments that look for changes after the extinction tests are depicted in Figure 11 and the main results of the experiments studying the effects of specific manipulations are depicted in Figure 12. We may conclude that the extinction process during the incubated seeking exhibits differences in dmPFC, vmPFC, DLS, DMS, core, shell and CeA activation, but not in OFC nor in BLA.

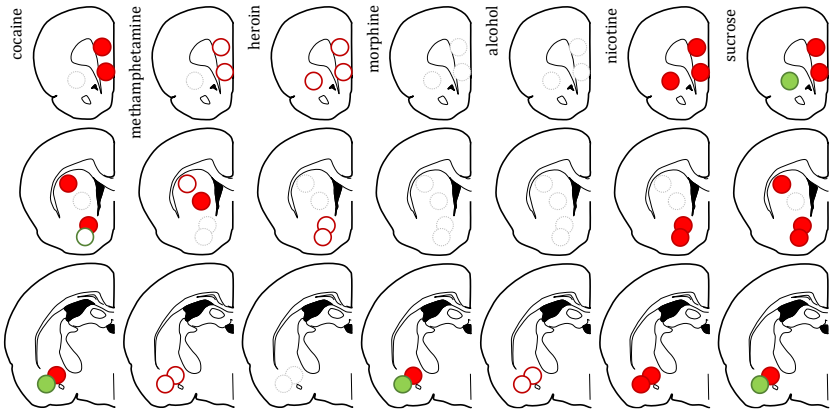


Figure 11. Level of evidence of the involvement of certain regions regarding the effects of extinction test.
Red: involved;
green: not involved;
filled: strong evidence;
empty: weak evidence;
grey: no evidence.

However, a region that intervenes in extinction does not have to be the promoter of an increased seeking, even if the former is a *sine qua non* condition. Therefore, it may be more pertinent to look at manipulation studies. With regard to these, we can conclude that there is a different effect on the incubated seeking if manipulations are aimed at the DMS (only tested with methamphetamine) and CeA, but not in dmPFC, vmPFC, OFC, DLS, core, shell or BLA. The implication of the latter in the

phenomenon of the incubation of the seeking will, therefore, be specific of substance and/or paradigm.

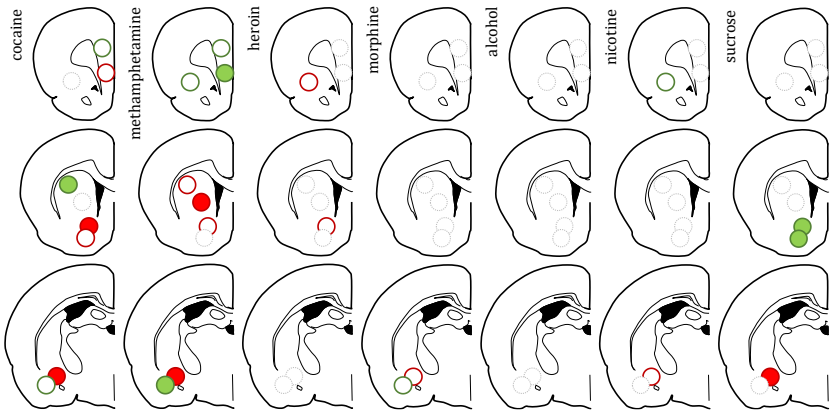


Figure 12. Level of evidence of the involvement of certain regions regarding the effects of manipulations.
Red: involved;
green: not involved;
filled: strong evidence;
empty: weak evidence;
grey: no evidence.

As we discussed earlier, dopamine seems to have a permissive role regarding the expression of the seeking incubation. Therefore, it is plausible that the CeA>SNpc> DMS pathway is the one involved in the incubation of the seeking. As the DMS region has only been studied with methamphetamine and the manipulation of SNpc does not affect the incubation of cocaine seeking, also a psychostimulant, the explanation with greater parsimony is that CeA is the region involved in the incubation of seeking in a general way.

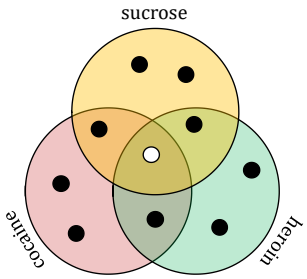
The implication of this region of the amygdala coincides with the results observed in fMRI studies with heroin addicts or with people subjected to different diets. In the study with heroin addicts, the authors observed that the amygdala (without differentiating between regions) became more active in the case of cues associated with heroin in long-term abstiners (Li *et al*., 2013). In the study with different diets the authors reported that the less effective diet to decrease craving was associated with a lower functional connectivity of the dlPFC with the amygdala when food-related cues are presented to the subjects (Kahathuduwa *et al*., 2018). Therefore, it seems that the central amygdala becomes more reactive with the cues associated with the substance as the abstinence progresses, and that this could be due, in part, to a lower cortical control.

In addition, levels of stress or anxiety are able to modulate the intensity of the incubation of seeking, although it is not clear to what extent these processes are essential for the phenomenon to occur.

HYPOTHESIS AND GOALS

On the basis of the literature discussed in the Introduction, The ***hypothesis*** of the PRESENT investigation is that the incubation of seeking has a common substrate shared by all reinforcers, as some authors argue, and that this substrate is found in the amygdala, the medial prefrontal cortex and/or the nucleus accumbens.

The ***general goal*** of this Doctoral Thesis was to look for biochemical alterations in the brain of the rats after the incubation of seeking. Since this phenomenon develops as abstinence progresses it is also accompanied by other effects of protracted abstinence specific to each substance. Therefore, the strategy that we followed was to look for *common changes after the protracted withdrawal* from three substances with very different pharmacological effects such as cocaine (psychostimulant), heroin (opioid) and sucrose (natural reinforcer). The potential common alterations found will therefore reflect effects related to the incubation of seeking and not primary pharmacological effects induced by the substances themselves.



On the basis of the previous knowledge on their role in the incubation process, we chose six brain regions to analyse: the shell and core regions of the nucleus accumbens, the basolateral amygdala, the central nucleus of the amygdala and the dorsal and ventromedial regions of the prefrontal cortex. We focused on molecular targets that would account for both plasticity-related effects and for basal activity of the region studied.

1st Goal:

To search for behavioural parameters during self-administration sessions that could be predictors of the development of the incubation of seeking, and then to look for correlations between them and biochemical parameters.

2nd Goal:

By using Western blot, qPCR, capillary electrophoresis and immunofluorescence, to search for changes during the incubation of seeking in parameters of the glutamatergic, GABAergic and endocannabinoid systems.

During sample dissection, we observed a noticeable adrenal hyperplasia in the animals that consumed cocaine and heroin. Due to the possible influence of stress in the incubation of seeking, we decided to extend the initial goals of the research:

3rd Goal:

To look for changes in parameters related to stress by Western blot, qPCR and radioimmunoassay that may be related to the incubation of seeking phenomenon.

PLATE XX.—*Erythroxylon coca* (Coca). (From Jackson: *Experimental Pharmacology and Materia Medica.*)

2. MATERIALS AND METHODS

2.1. JUSTIFICATION OF THE MODEL AND THE SAMPLE PROCESING

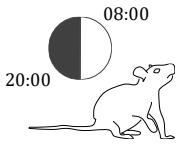
Justification of the model. To perform the study we chose to use young adult male rats of the Lewis strain. This strain of rat has been used in our laboratory for years, and there were no incubation studies where this strain had been used. Most of the published studies employed Long-Evans or Sprague-Dawley, and to a lesser extent rats of the Wistar strain. Therefore, we reasoned that it would be interesting to use this strain to increase the diversity of strains in which the incubation phenomenon has been studied and hence enhance the validity of the published results. On the other hand, we use male animals, and not females. Although the incubation phenomenon with female rats is currently being studied in our laboratory we decided to focus on males in this Thesis to ensure that we would be able to compare the results obtained with those in the literature. The preference for males, in addition to the obvious bias that exists in Science, is due to the fact that the majority of cocaine and heroin addicts in our society are men. Finally, young adult rats were chosen according to the age at which these substances are consumed.

According to the literature, in order to observe the incubation of seeking of cocaine and heroin it is necessary (although there are exceptions) to use extended access protocols, which usually promote escalation in drug intake. Quite surprisingly, short-access protocols are sufficient to induce incubation of seeking with sucrose (Grimm *et al*., 2002). Since our goal was to study the incubation of seeking and not the escalation, we decided to use protocols for drugs of abuse (extended access) and for sucrose (restricted access) that were not identical in terms of access lenght. Furthermore, it has recently been published that a paradigm of extended access to natural reinforcers with high caloric content induces compulsive and impulsive phenotypes, among other characteristics (Burokas *et al*., 2018).

Justification of sample processing. The goal of this Doctoral Thesis was to describe biochemical changes that could be related to the phenomenon of seeking incubation. To do this, six regions shown in the literature to be related to the phenomenon were analysed. Our working hypothesis is that the incubation of the seeking of the different substances has a common biological substrate, so we looked for changes during the withdrawal from three substances that could be interconnected. In order to optimise the samples obtained from each subject, the samples were dissected and processed in such a way that from the same biological sample, protein expression levels (by Western blot), amine levels (by capillary electrophoresis) and levels of gene expression (by qPCR) could be assayed. In addition, thin tissue slices were obtained during cryostat sectioning that would then be analysed by immunohistochemistry, immunofluorescence or *in situ* hybridization.

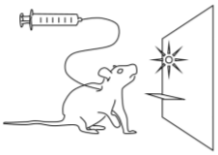
The drawback of such approach was that the analysis of amine content was not possible for all the samples, and there were a few samples in which, due to its instability, RNA was lost. Even so, we believe that it is the optimal way to proceed, as this incurs in the reduction of animals that are used in the experiments (by 50% at least) and allowed us to perform multivariant analysis with variables of different nature.

2.2. ANIMAL PROCEDURES



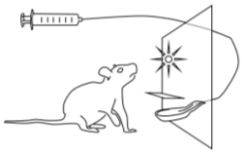
Light:dark cycle. ON at 08:00, OFF at 20:00.

Animals. Male rats of the Lewis strain were used (Harlan International Ibérica, N=109), between 300-320g of weight at the beginning of the experiments. The animals were kept in the vivarium in a light-dark cycle (on at 08:00 am), at a constant temperature (20±2°C), with water and food (Panlab, commercial diet for rodents A04/A03) *ad libitum*. From the moment of their arrival, the animals were housed in groups of three until they underwent surgery a week before begining the self-administration sessions. Then they were single-housed to prevent the rats from biting the catheters of their cage mates. The animals that did not need operation were also single-housed at the same time. All animals were maintained and handled in accordance with the European Union Laboratory Animal Care Standards (2010/63/EU).



SCH experiment. Schedule with the main components.

Surgery. Both the rats that self-administered cocaine and intravenous heroin and their respective controls (saline group) were subjected to catheter implantation one week before the first self-administration session (*surgery by Rosa Ferrado*). The rats that consumed sucrose and their controls (water group) drank from spoons made *ad hoc* (*manufactured by Alberto Marcos*) so they did not need any surgery. The catheter was implanted into the left jugular vein approximately at the level of the atrium, continuing subcutaneously until emerging in the medial scapular region. This operation was performed under anaesthesia with isoflurane (5% in induction, 2% in maintenance) and with analgesia (buprenorphine). In order to maintain functional catheters and prevent infections, 0.5mL of saline with heparin and gentamicin (30mg/mL) was injected daily through them (Higuera-Matas *et al*., 2008).



WZ experiment. Schedule with the main components.

Experimental batches. Two experimental batches were carried out, both undergoing ten days of self-administration. A first batch was used to validate the incubation model in our laboratory (*Experiment 1*). After one day (wd1) or one month (wd30) of forced abstinence, the animals were subjected to an extinction test to measure the seeking. Some animals underwent both extinction tests, allowing us to calculate their incubation coefficients. A second batch, which did not go through the extinction tests to avoid possible effects caused by the extinction learning, was used to perform the biochemical analyses (*Experiment 2*). The rats underwent ten days of self-administration and were sacrificed after one day (wd1) or one month (wd30) of forced abstinence. Two batches were carried out in parallel: the experiment with drugs, composed by wd1 and wd30 groups for saline, cocaine and heroin (six groups, *n*=8 rats per group, *SCH experiment*) and the experiment with water and sugar (four groups, *n*=9 rats per group, *WZ experiment*).

Burrhus Frederic Skinner, 1904-1990.

Self-administration sessions. Both experiments (SCH and WZ) have in common that they were performed in Skinner boxes of operant conditioning (Coulburn and MedAssociated), monitored by the MedPC software. The solutions of cocaine, heroin, saline, water and sugar water were contained by syringes located in pumps on the boxes, connected to their destination (the catheter in the case of the SCH experiment or the spoon in the case of the WZ experiment) using a plastic tubing. The light of the camera was on until the beginning of the session, at which time it turned off (although the door was slightly open to maintain the light cycle), and turned on again at the end of the session. Inside the box there were two levers: one active (lever 1) and one inactive (lever 2). Each time the animal pressed the active lever the pump was activated, which for 5 seconds administered the rat the corresponding dose of substance (unconditioned stimulus, US), while a led was lit over the lever for 10 seconds (conditioned stimulus, CS). After 5 seconds of administration a time out of 40 seconds ensues, during which the animal received neither US nor CS no matter how much the lever was pressed. The rats of the experiment SCH were subjected to extended access sessions (6h/day, Airavaara *et al*., 2011; Conrad *et al*., 2008), and the rats of the experiment WZ to restricted access sessions (2h/day, Harkness *et al*., 2010). Doses per injection were 0.075mg/kg of heroin, 0.75mg/kg of cocaine-HCl, or sucrose (Sigma-Aldrich S1888) 10% w/v diluted in an average of 0.1mL of saline (ERN, Vitulia physiological saline, 0.9% w/v) or tap water.

Missing animals. In total 25 subjects were lost from the experiments with intravenous administration due to problems with the catheter.

Abstinence and extinction test. The first extinction test was performed the day after the last self-administration session, and the second test one month later. Meanwhile, the rats were regularly handled by the experimenter. The extinction test was similar to a self-administration session with the difference that, despite receiving the CS, the rats did not receive the US (the substance). It lasted 3 hours in the case of cocaine and heroin and 2 hours in the case of sucrose.

Behavioural parameters. In each session, for both self-administration and extinction, the following parameters were obtained:

block 1: Direct parameters

· **LP1**: number of lever 1 presses during the time this lever is active.
Because it is a fixed ratio 1 schedule, LP1 number is equivalent to the number of doses.
· **LP2**: number of lever 2 presses during the time lever 1 is active.
· **LPTOR1**: number of lever 1 presses during the time out.
· **LPTOR2**: number of lever 2 presses during the time out.

block 2: Extracted parameters
(by Eduardo Ucha, Marcos Ucha and Raquel Santos)

· **Latency**: delay between the initiation of the session and the first lever 1 press.
· **T1/2**: time until the animal reaches half of the LP1 of the entire session.
The lower this value, the more concentrated the lever 1 presses are in the beginning of a session.
· **Interval**: time between two lever 1 presses in a session
· **Average of intervals**: average of the intervals of a session.
· **Median of intervals**: median of the intervals of a session.
The lower these two values, the higher the frequency of lever 1 presses.
· **Asymmetry of intervals**: asymmetry coefficient of the intervals distribution.
The higher this coefficient, the higher the frequency of lever 1 presses.
· **LP30**: number of lever 1 presses during the first 30min of a session.
· **LP60**: number of lever 1 presses during the first hour of a session.

block 3: Derived parameters

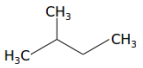
· **L1t** = LP1 + LPTOR1.
· **L2t** = LP2 + LPTOR2.
· **Lt** = L1t + L2t.
· **LP301**: LP30 divided by the average number of lever 1 presses in 30min.
· **LP601**: LP60 divided by the average number of lever 1 presses in one hour.
The higher these two values, the more concentrated the lever 1 presses are at the beginning of a session.
· **LP1t**: sum of the LP1 values of the 10 sessions.
· **'*parameter*'i:** average of the value of a parameter during the first 3 sessions (initial).
· **'*parameter*'f:** average of the value of a parameter during the last 3 sessions (final).
· **d'*parameter*'** = '*parameter*'f - '*parameter*'i.
· **r'*parameter*'** = '*parameter*'f/'*parameter*'i.

In animals that underwent both extinction test, wd1 and wd30, incubation coefficients were calculated:

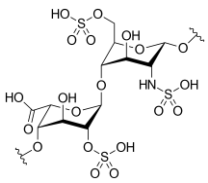
block 4: Incubation coefficients

· **incu_d'*parameter*'**: difference (wd30 - wd1) of LP1, L1t and Lt.
· **incu_r'*parameter*'**: ratio wd30/wd1 of LP1, L1t and Lt.

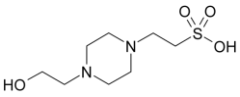
2.3. SAMPLE PROCESING



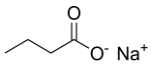
Isopentane



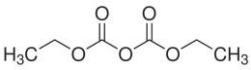
Heparin



4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
(HEPES)
pK_{a1}=3.0; pK_{a2}=7.5



Sodium butyrate.
Demethylases inhibitor.



Diethyl pyrocarbonate
(DEPC).
It inhibits RNases by covalently binding histidine residues (to a lesser extent, to lysine, tyrosine and cysteine residues). When it is autoclaved it releases ethanol and CO_2.

Animal sacrifice. The animals were weighed and sacrificed after one or 30 days of abstinence 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 (-78.5°C), and stored at -70°C. In addition, blood was collected from the trunk in a heparinized tube and the plasma was extracted after centrifugation at 1000*g* for 10min at 4°C and stored at -70°C. Adrenal glands, spleen and liver were removed. The structures were weighed and stored at -70°C (only small fragments were stored in the case of spleen and liver) (*with the help of Gonzalo Moreno, Luis Carrillo and Rosa Ferrado*).

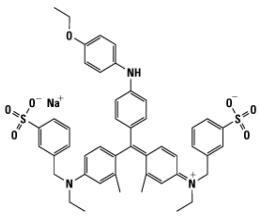
Reagents and buffers. HEPES (Sigma H3375), sucrose (Sigma S1888), protease inhibitors (Roche complete EDTA-free 11.873.580.001), phosphatase inhibitors (Roche PhosSTOP 04.906.837.001), sodium butyrate (Sigma B5887).
·Q buffer: 50mM HEPES, pH 7.5, 320mM sucrose, protease and phosphatase inhibitors, 20mM sodium butyrate, in DEPC-water.

Brain dissection. One hour before being dissected, brain was tempered at -20°C in the cryostat chamber (Microm, Cryostat HM 500O) embedded in TissueTek (Sakura, 4583). 300µm thickness slices were collected on a sterile cold surface and dissected with a lancet, according to a rat neuroanatomical atlas (Paxinos and Watson, 2007). The tissue was stored during dissection in sterile tubes of 0.2mL in dry ice and then at -70°C. 40µm thickness slices were also made from the same brains, at different regions, which were placed on slides for adhesion (Thermo Scientific, SuperFrost® Plus, Menzel Gläser). After air-drying, they were stored until use at -30°C.

Missing samples. Two brains were lost ("*water*" group) due to storage problems.

Tissue homogenization. Samples were weighed and homogenized in Q buffer using a motorized mortar (Sigma-Aldrich, pellet pestle Z359971). After being kept on ice for 10min they were centrifuged at 1000*g* for 10min at 4°C. The pellet was separated from the supernatant, resuspended in Q buffer and the supernatant was divided into three different tubes. One of the tubes contained 800µL of QIAzol (Qiagen 79306) and the volume equivalent to 3-4mg of original tissue (≤80µL) was added for extraction of cytoplasmic RNA. Another tube contained loading buffer for Western blot. A portion of the resuspended pellet was also separated with loading buffer. The third tube was empty and was used for capillary electrophoresis and protein quantification. All samples were stored again at -70°C.

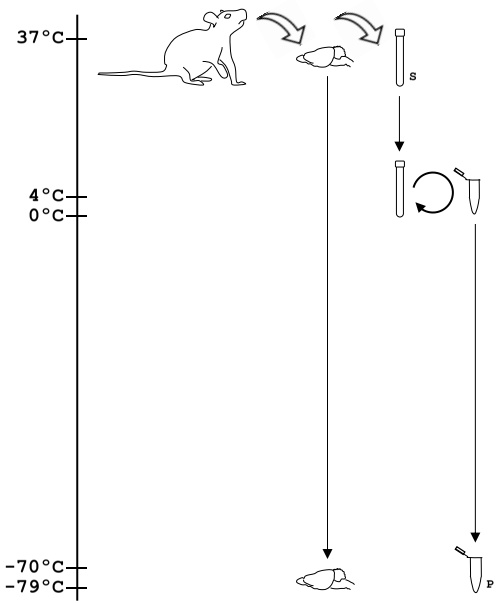
2.4. PROTEIN QUANTIFICATION



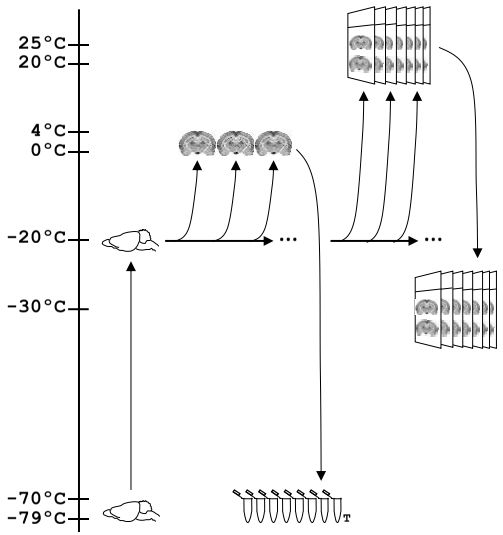
Coomassie blue G-250
(Bradford reagent)
λ_{free}=465nm
λ_{bound}=595nm

Protein quantification. The protein content was quantified in 96-well plates (Falcon, Microtest 96, 35-3072) using the colorimetric method described by Bradford (1976) with slight modifications. Briefly, 250µL of reagent (1:5 dilution of Biorad Protein Assay Dye Reagent Concentrate-treat 500-0006) was added to 5µL of sample. In parallel, a calibration curve was made (r^2≥0.99) using bovine serum albumin (BSA, Sigma A9647) from 0.05 to 1.5µg/µL. The absorbance ratio 595nm/450nm (measured in the Asys Hitech DigiScan plate reader) was used to interpolate the values.

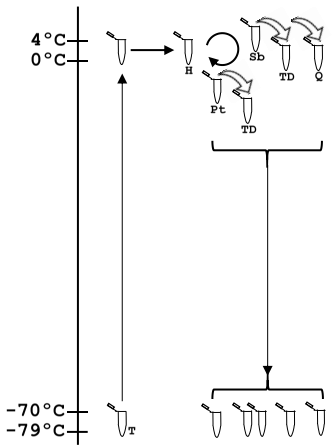
Schedule of sample procesing ⟶



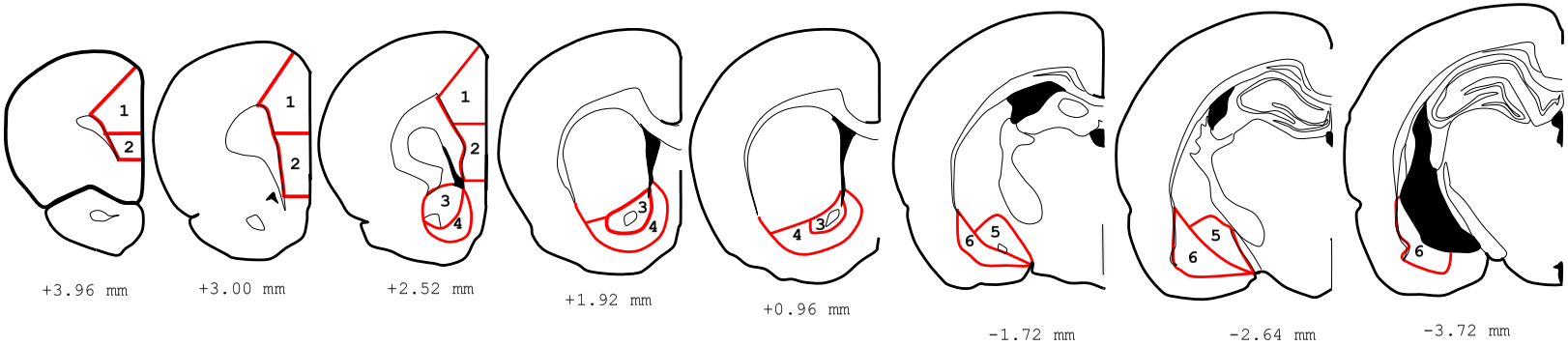
Protocol during the sacrifice.
After the sacrifice of the rat, brain is extracted and plasma (P) is separated from total blood (S). Samples are stored at -70ºC.



Protocol during the brain dissection.
Brain is tempered in the cryostat chamber (-20ºC) and 300µm thick slices are performed. Dissected tissue regions (T) are stored at -70ºC. In parallel, 40µm thick slices are performed. After air-drying, they are stored at -30ºC.



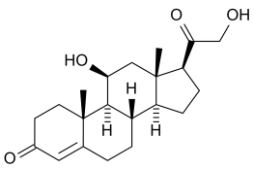
Protocol during homogenization.
Tissue (T) is thawed and homogenized (H) on ice, then centrifuged at 1000*g* separating a pellet (Pt) and a supernatant (Sb). Different aliquots are prepared, with loading buffer (TD), QIAzol (Q) or nothing.



[1] dmPFC
[2] vmPFC
[3] NAc core
[4] NAc shell
[5] CeA/MeA
[6] BLA

2.5. RADIOIMMUNOASSAY (RIA)

(with the counselling of Emilio Ambrosio)



Corticosterone

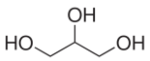
Danger:
radioactivity.

Radioimmunoassay. The plasmas were thawed, centrifuged at 10 000*g* for 10min at 4°C to discard precipitates and, after tempering them at room temperature (RT), the RIA was started using the Coat-a-Count® Rat Corticosterone kit (Siemens). Briefly, 50µL sample or calibrator is added to tubes covered with anti-corticosterone antibody. Then 1mL of I^{125}-corticosterone preparation is added, vortexed and incubated at RT for 2h. The content is decanted and the signal is analysed (1min/tube, in counts per minute, *cpm*) in a gamma counter (Wallac, Automatic Gamma Counter 1470 Wizard). To estimate the non-specific binding (NSB), non-covered tubes, but with sample or calibrator, are measured. The maximum binding value (MB) is estimated by measuring a calibrated tube without labelled corticosterone.

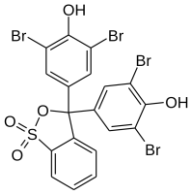
Analysis. The corticosterone values are interpolated in a semilogarithmic line facing the logarithm of the concentration with the binding value (B), and is normalized with respect to the volume of plasma:

$$B = \frac{cpm_{sample\ or\ calibrator} - cpm_{NSB}}{cpm_{MB}}$$

2.6. WESTERN BLOT

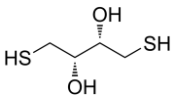


Glycerol
To increase the density of the sample.
density=1.26g/cm^3

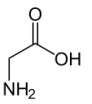


Bromophenol blue
To color the sample.
pH<3.0: yellow
pH>4.6: violet

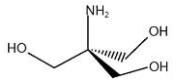
Reagents and buffers. Sodium dodecylsulphate (SDS, Sigma L3771), bromophenol blue (BrPhB; General Electric 17-1329-01), glycerol (Panreac 141339.124), sodium chloride (NaCl, VWR 27800.360), Tris (VWR 28811.295), 2,2,2-trichloroethanol (TCE, Sigma T54801), Tween20 (Sigma P9416), TEMED (Biorad #161-0800), ammonium persulfate ($(NH_4)_2S_2O_8$, PSA; Biorad #1610700), glycine (Fisher G/P460/53), acrylamide:bis 30% 1:29 (Biorad #161-0156), dithiothreitol (DTT; Sigma), β-mercaptoethanol (βME; Sigma), anti-PSD95 (Cell Signaling #2507), anti-rabbit IgG HRP-linked (Abcam ab6721), anti-gephyrin (Cell Signaling #14304), ECL 2 (Thermo Scientific Pierce® #1896433A&B).
·*Stacking gel*: 125mM Tris, pH 6.8, 0.1% w/v SDS, 4% w/v acrylamide:bis (1:29), 0.5% v/v TCE.
·*Running gel*: 375mM Tris, pH 8.8, 0.1% w/v SDS, 12% w/v acrylamide:bis (1:29), 0.5% v/v TCE.
·*Electrophoresis buffer*: 25mM Tris, 192mM glycine, 0.1% w/v SDS.
·*Loading buffer x6*: 375mM Tris, pH 6.8, 60% v/v glycerol, 12% w/v SDS, 300mM DTT, 0.012% w/v BrPhB.
·*TBST*: 50mM Tris, pH 7.5, 150mM NaCl, 0.1% v/v Tween20.
·*Stripping buffer*: 62.5mM Tris, pH 6.8, 2% w/v SDS, 0.8% v/v βME.



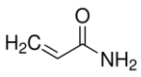
Dithiothreitol (DTT).
Reducing agent.



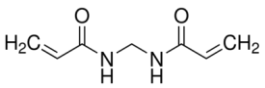
Glycine (Gly)
pK_{a1}=2.35;
pK_{a2}=9.78



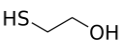
2-Amino-2-(hydroxymethyl)propane-1,3-diol
(Tris)
pK_a=8.06



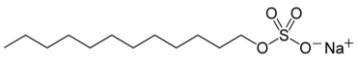
Acrylamide.
Monomer that gives linear polymers.



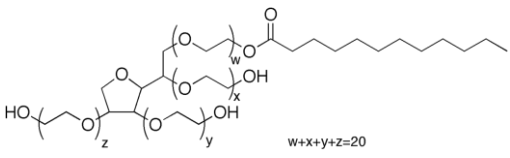
Bisacrylamide.
Monomer that links linear polymers to each other.



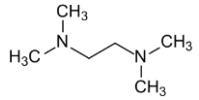
β-mercaptoethanol.
Reducing agent.



Sodium dodecylsulphate (SDS).
Detergent used to denature proteins and endow them with negative charge.

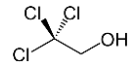


Polyethylene glycol sorbitan monolaurate (Tween 20).
Detergent used to avoid nonspecific binding.

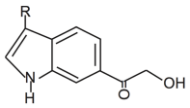


N,N,N',N'-tetramethylethane-1,2-diamine
(TEMED).
Polymerizing agent.

Western blot. 5µg of protein from each sample was loaded into MiniProtean® Tetra System (Biorad) in 12% SDS-PAGE (SDS-polyacrylamide gel electrophoresis) hand-made gels. For the quantification of total protein as load control, the gels were exposed to ultraviolet light for one minute to activate the reaction between the TCE and the tryptophan residues (Ladner *et al*., 2004), and then digitized (reading at 312nm). Proteins were transferred with Trans-Blot® Turbo transfer system (Biorad) to 0.2µm PVDF membranes (Trans-Blot® Turbo ™ transfer pack, Biorad), and blocked with 5% w/v BSA in TBST for one hour at RT. The membranes were incubated overnight in TBST at 4°C with anti-PSD95 (1:3000). They were then washed in TBST (3x15min), incubated with secondary antibody (1:5000) for one hour at RT and washed thrice again. The HRP enzyme was exposed to the ECL2 substrate following the manufacturer's recommendations. Stripping was performed to quantify gephyrin (1:2000) in the same membranes. Briefly, the membranes were incubated in stripping buffer at 50°C for 45min, washed in TBST (4x15min) and re-blocked. All incubations were performed under agitation.

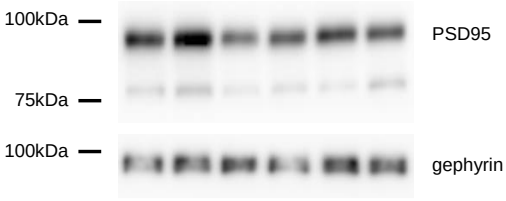


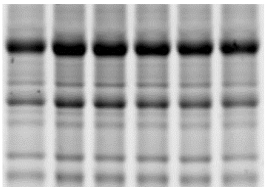
Trichloroethanol
(TCE)



Fluorophore resulting from the reaction of the indole group of the tryptophan residues with TCE.

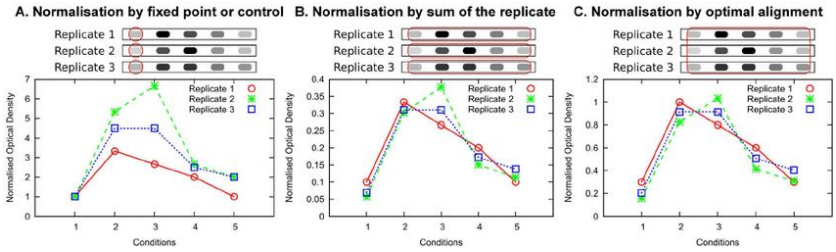
Example of the bands obtained from PSD95 and gephyrin.





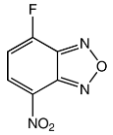
Example of gel stained with TCE-UV.

Analysis. All the scans were performed on an Amersham Imager 600 (General Electrics) system. The proteins were analyzed by densitometry using the Fiji (ImageJ) free software. Due to the number of samples, several gels were needed for each region. There were always, in each gel, samples from all experimental groups, and they were prevented from always being located in the same wells or in adjacent wells. To normalize the data, first each variable (total protein, PSD95 and gephyrin) was normalized with respect to its average in each gel and then the ratio of each protein was calculated with the total protein value for each individual (Degasperi *et al*., 2014). The PSD95/gephyrin ratio was also calculated.

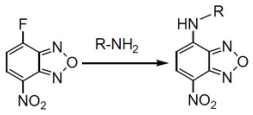


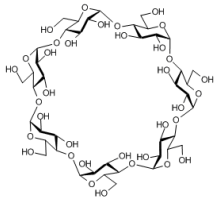
Normalisation. The chosen option was the sum of biological replicates (B). Degasperi *et al*. (2014)

2.7. CAPILLARY ELECTROPHORESIS

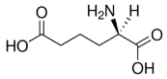


4-fluoro-7-nitro-benzofurazan (NBD-F)
$\lambda_{excitation}$=470nm
$\lambda_{emission}$=530nm





β-cyclodextrin.
Its funnel-shaped structure allows the separation of the L and D forms, by retaining a form with more affinity than another.



L-2-aminodipidic acid
(internal standard; 14)

Reagents and buffers. From Sigma: L-glutamic acid (49449), glycine (G7126), L-glutamine (49419), taurine (T0625), L-serine (S4500), D-serine (S4250), γ-aminobutyric acid (GABA, A5835), L-2-aminodipidic acid (A7275) as internal standard (IS), L-proline (81709), L-isoleucine (W527602), L-ornithine (O2375), L-threonine (T8625). β-cyclodextrin (βCD) (SAFC W40,282-6), 4-fluoro-7-nitro-benzofurazan (NBD-F; Alfa-Aesar J61336).
·*Tissue derivatization solution*: 25µL diluted sample, 10µL 100mM IS, 25µL 20mM NBD-F, 150µL 10mM borate buffer pH 9.0.
·*Plasma derivatization solution*: 20µL sample, 20µL 200mM IS, 25µL 40mM NBD-F, 150µL 10mM borate buffer pH 10.0.
·*Tissue electrophoresis buffer*: 90mM borate buffer pH 10.25, 12.5mM βCD.
·*Plasma electrophoresis buffer*: 175mM borate buffer pH 10.25, 12.5mM βCD.

Capillary electrophoresis. (*by Alberto Marcos except sample preparation*) The content of amines was analysed in those samples in which there was enough tissue, according to the method described in Lorenzo *et al*. (2013a). The content of amines was analysed in all plasma samples, according to the method described in Lorenzo *et al*. (2013b). For tissue analysis, 5µl of 1:5 supernatant was diluted in 10mM borate buffer pH 9.0. The plasma was not diluted. Samples (supernatants and plasmas) were passed through a 0.22µm filter (VWR 28145-491 13mm syringe filter 0.2µm PTFE membrane) and then stored at -70°C until use. In parallel, cocktails of different amines were prepared to be used as a calibration line. L-2-aminodipidic acid was used as internal standard (IS). In each cocktail the concentration of each amine was different but the total concentration of amines was maintained. The derivatization was carried out at 60°C for 15min 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 30min before the start of the electrophoresis, carried out at 17°C. Before its first use, the capillary column of silica (length: 60cm, inner diameter: 75µm) was conditioned with 1M NaOH (15min) and water (15min), and between electrophoresis with 0.1M HCl (3min), water (5min) and electrophoresis buffer (5min). The sample was injected at the anode at 0.5psi (33mbar) for 10 seconds. A potential difference of 21kV was applied, observing currents of 78µA (supernatants) and 120µA (plasmas). The molecules were detected at the cathode by means of laser induced fluorescence (LIF), exciting at 488nm (argon lamp) and detecting the emission at 522nm. To obtain reproducible electropherograms, the electrophoresis buffer was renewed every six analyses. In each session a calibration curve was also injected.

Analysis. The electropherograms (Figure 13) were analysed with the 32 Karat™ software, using the corrected area normalized to the internal standard. The values were expressed as pmol of amine per µg of protein (supernatants) or pmol of amine per mL (plasma). Different ratios related to activity balances were also calculated (L-glutamate/GABA, L-glutamate/D-serine, L-glutamate/glycine) and with replacement rates (L-glutamate/L-glutamine, D-serine/L-serine, L-glutamine/L-ornithine).

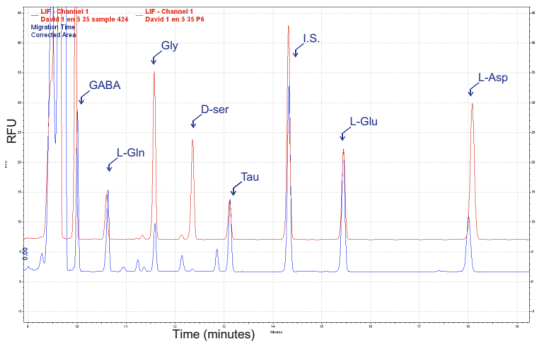
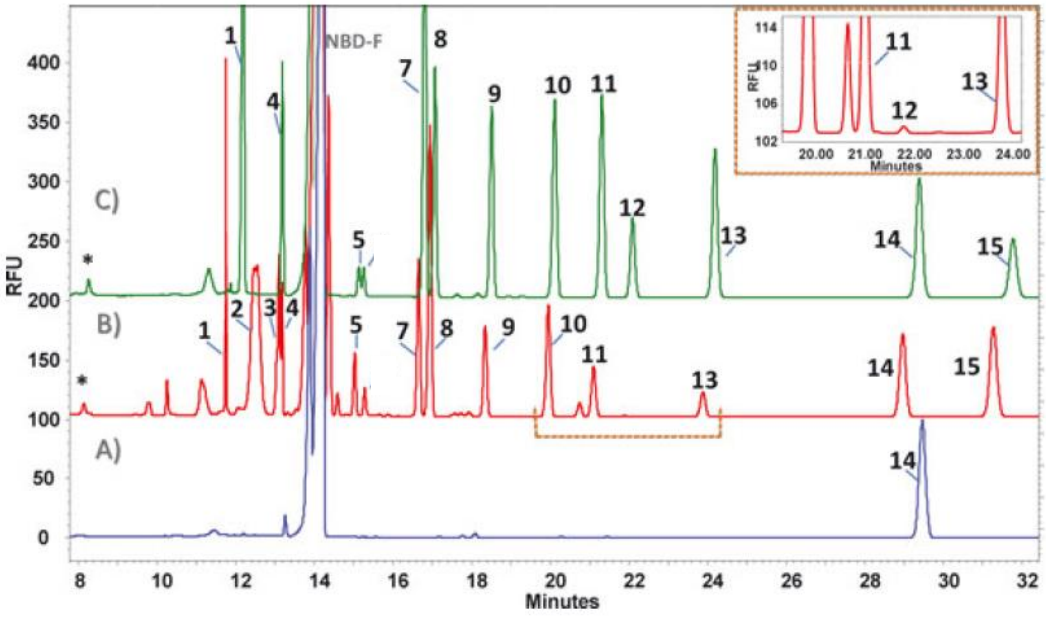
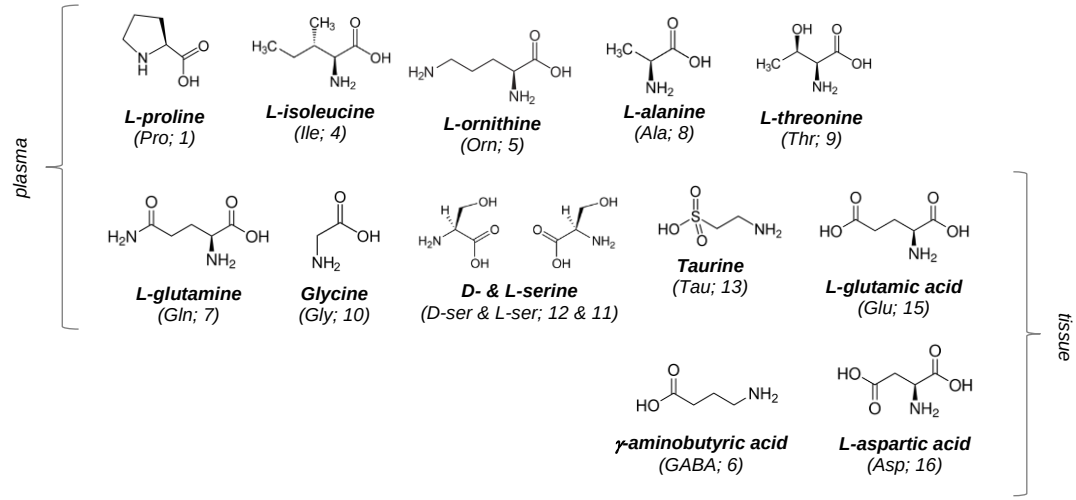
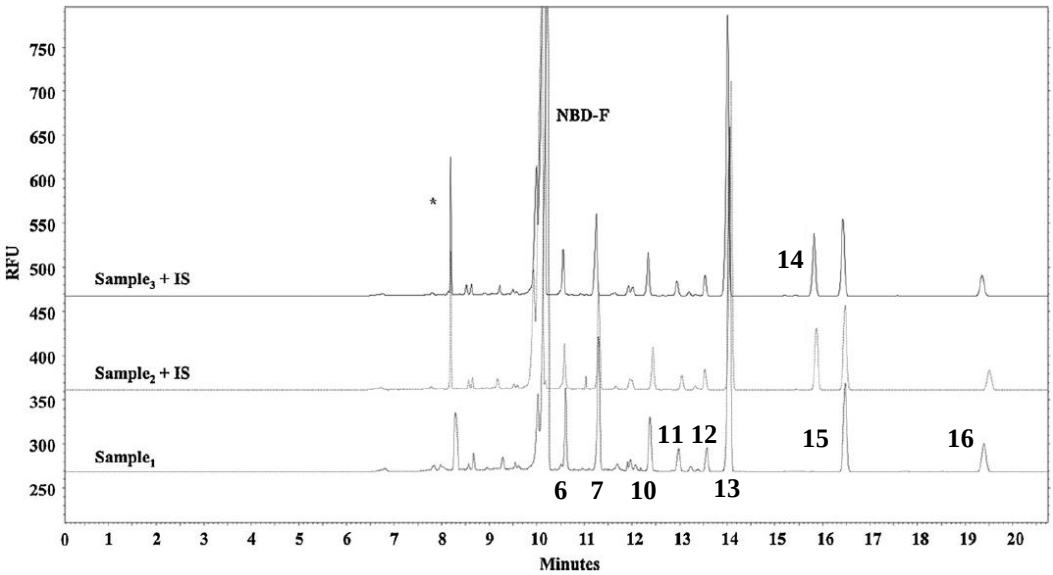


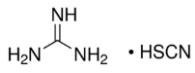
Figure 13. *Right, this page*: example of electropherogram obtained in our laboratory. *On the following page*: two type electropherograms are presented, from a plasma sample (*above*) and from a sample of brain tissue (*below*). Although they share most of the analytes, both samples also have different amino acids.Lorenzo *et al*. (2013a and b).


NBD-F
C)
B)
A)
RFU
Minutes

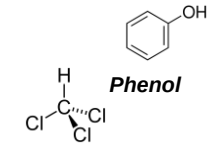

plasma
tissue
L-proline
(Pro; 1)
L-isoleucine
(Ile; 4)
L-ornithine
(Orn; 5)
L-alanine
(Ala; 8)
L-threonine
(Thr; 9)
L-glutamine
(Gln; 7)
Glycine
(Gly; 10)
D- & L-serine
(D-ser & L-ser; 12 & 11)
Taurine
(Tau; 13)
L-glutamic acid
(Glu; 15)
γ-aminobutyric acid
(GABA; 6)
L-aspartic acid
(Asp; 16)


NBD-F
Sample3 + IS
Sample2 + IS
Sample1
RFU
Minutes

2.8. GENE EXPRESSION



Guanidine thiocyanate.
Chaotropic agent.



Phenol

Chloroform

Isopropanol

RNA isolation. The RNA from the supernatant of 1000*g* (cytoplasm) was extracted from the equivalent to 3-4mg of tissue, using a protocol adapted from the method described in Chomczynski and Sacchi (2006, 1987). Briefly, the sample that had been stored with QIAzol (containing phenol and guanidine thiocyanate) was thawed and left at RT for at least 5min to destabilize the nucleoprotein complexes. 160µL of chloroform (Merck 1.02445.2500) was 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 1.5mL tube, with 400µL of isopropanol (Fischer Scientific, BP2618) and 10µg of glycogen as carrier (Sigma G1767), mixed and incubated at RT for 10min. The lower (organic) phase was stored at -70°C in case it was necessary to extract DNA or protein from it. It was centrifuged at 12 000*g* for 10min at 4°C and the supernatant discarded. The precipitate was washed twice in 1mL of cold 75% v/v ethanol (-20°C), centrifuging at 7500*g* for 5 min at 4°C and discarding the supernatants. Finally, the precipitate was allowed to air-dry and resuspended in nuclease-free water. It was stored at -70°C.

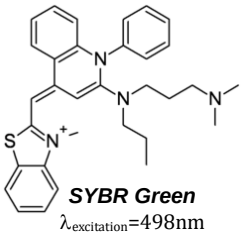
RNA quantification. The RNA was quantified in a Bioanalizer 2100 system (Agilent, RNA Nano Chips, 5067-1511), accepting values of RIN≥7.0. Unless exceptions, absorbance ratios 260nm/280nm≥1.8 were obtained. An isolation efficiency of 0.7-1.0µg RNA/mg tissue was obtained.

*******carrier*: molecule of a nature similar to the target, used when the start material is limited. tRNA, linear acrylamide or glycogen are usually used to precipitate nucleic acids.

Retrotranscription. An amount of RNA (from 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.

*******RIN* (*RNA Integrity Number* value between 0 and 10 that denotes the integration of the RNA and is calculated based on an algorithm by Agilent Bioanalyzer.

qPCR. (*with the collaboration of Inmaculada Ballesteros-Yáñez, Carlos Alberto Castillo and Marcos Ucha*) Gene expression was analysed using SYBR Green (Biorad, SsoAdvanced Universal SYBR Green Supermix) as a fluorophore, in a CFX96 C1000 Touch of Biorad system, with 96-well plates (Biorad, Hard-Shell PCR Plates 96-Well WHT/WHT, HSP9655) covered with transparent film (BioScience, Sorenson, µltraAmp Plate Seal, 36590). The total reaction volume was 10µL, and the primers were in the range of 500-750nM. The thermal protocol was as follows: 5min at 95°C (disinhibition of the polymerase), 40 cycles of 5min at 95°C (denaturation) and 30 seconds at 60°C (hybridization and polymerization). Finally, a melting curve from 60°C to 95°C was performed.



SYBR Green
$\lambda_{excitation}$=498nm
$\lambda_{emmision}$=522nm

Analysis. The Ct values were taken and the efficiencies (ε) were calculated with the free software LinRegPCR (Ruijter *et al*., 2009). *Gapdh* was used as housekeeping gene. The fold change for each sample and gene was calculated according to the method described by Pfaffl (2001):

$$Fold\ Change = \frac{\varepsilon_{target\ gene}{}^{(Ct_{control} - Ct_{sample})}}{\varepsilon_{Gapdh}{}^{(Ct_{control} - Ct_{sample})}}$$

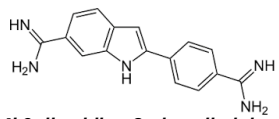
In addition to this *'target gene'/Gapdh* ratio, others were calculated: *Grin2a/Grin2b*, *Gria1/Gria2*, *Gabra1/Gabra2*, *Gabrg2/Gabrd*, *Dagla/Mgll*, *Napepld/Faah*. The sequence of the primers is presented in Table 5. They were designed *ad hoc* using the primerBLAST tool on the NCBI webpage in such a way that they could amplify any of the multiple transcripts in cases where there is more than one, based on the data published in the Gene database (also from the NCBI).

Table 5. Sequence of the primers.

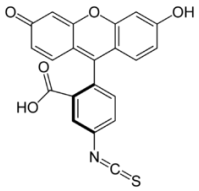
Identification	Complete name of the protein	Sequence of sense (S) and antisense (A) primers
	Housekeeping gene	
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase	S: 5'-tccctgttctagagacag-3' A: 5'-ccactttgtcacaagaga-3'
	Genes of subunits of glutamatergic ionotropic receptors	
NR1 *(Grin1)*	NMDA receptor, subunit 1	S: 5'-aacctgcagaaccgcaag-3' A: 5'-gcttgatgagcaggtctatgc-3'
NR2A *(Grin2a)*	NMDA receptor, subunit 2A	S: 5'-tgtgaagaaatgctgcaagg-3' A: 5'-gaacgctcctcattgatggt-3'
NR2B *(Grin2b)*	NMDA receptor, subunit 2B	S: 5'-caatggcagcacagagagga-3' A: 5'-agcaatgccatagccggtag-3'
GluA1 *(Gria1)*	AMPA receptor, subunit 1	S: 5'-agaggctggtggtggttgact-3' A: 5'-accctggtatggtctcggga-3'
GluA2 *(Gria2)*	AMPA receptor, subunit 2	S: 5'-ggcgtgtaatcctggactgt-3' A: 5'-acaccagggaatcgtcgtag-3'
	Genes of subunits of GABAergic ionotropic receptors	
$GABA_A\alpha1$	$GABA_A$ receptor, alpha 1 subunit	S: 5'-ttgactgtgagagccgaatg-3' A: 5'-aaacgtgacccatcttctgc-3'
$GABA_A\alpha2$	$GABA_A$ receptor, alpha 2 subunit	S: 5'-ccaatgcacttggaggactt-3' A: 5'-actggcccagcaaatcatac-3'
$GABA_A\gamma2$	$GABA_A$ receptor, gamma 2 subunit	S: 5'-cggaaaccaagcaaggataa-3' A: 5'-acagtccttgccatccaaac-3'
$GABA_A\delta$	$GABA_A$ receptor, delta subunit	S: 5'-gctggacctggagagctatg-3' A: 5'-ccgaagctggaagtgtaagc-3'
	Genes of the endocannabinoid system	
CB1 *(Cnr1)*	Cannabinoid receptor 1	S: 5'-gtcgatcctagatggccttgc-3' A: 5'-gtcattcgagcccacgtagag-3'
NAPE-PLD	*N*-acylphosphatidylethanolamine phospholipase D	S: 5'-accaacatgctgacccagag-3' A: 5'-atcgtgactctccgtgcttc-3'
FAAH *(Faah)*	Amidohydrolase of fatty acids	S: 5'-gttacagagtggagagctgtcc-3' A: 5'-gtctcacagtcggtcagatagg-3'
DAGLα *(Dagla)*	Diacylglycerol lipase alfa	S: 5'-ctttgctgaatttttccgtgacc-3' A: 5'-ttgtttgcctcatccagcac-3'
MAGL *(Mgll)*	Monoacylglycerol lipase	S: 5'-ctacctgctcatggaatc-3' A: 5'-gacacccacgtatttatttc-3'
	Gene related to dendritic spines	
β-actin *(Actb)*	β-actin	S: 5'-gcatcctgaccctgaagtaccc-3' A: 5'-gtgttgaaggtctcaaacatgatctgg-3'
	Genes of adrenergic receptors	
$\alpha1$ *(Adra1)*	Adrenoceptor alpha 1	S: 5'-ctcgagagaagaaagctgcca-3' A: 5'-aaaacggtttccgaaggcttg-3'
$\alpha2A$ *(Adra2a)*	Adrenoceptor alpha 2A	S: 5'-tcctgagagggaagggattt-3' A: 5'-agttactggggcaagtggtg-3'
$\beta1$ *(Adrb1)*	Adrenoceptor beta 1	S: 5'-gctctggacttcggtagacg-3' A: '-acttggggtcgttgtagcag-3'

2.9. PERINEURONAL NETS

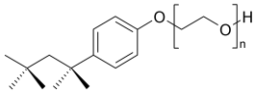
(staining and microscopy by Paula Díez, as well as SCH experiment counting; microscope kindly provided by Dr. Javier de Felipe)



4',6-diamidino-2-phenylindole (DAPI)
$\lambda_{excitation}$=358nm
$\lambda_{emmision}$=461nm



Fluorescein isothiocyanate (FITC)
$\lambda_{excitation}$=492nm
$\lambda_{emmision}$=518nm



Triton X-100

Reagents and buffers. Phosphate salts, Triton X-100, 37% w/v formaldehyde, WFA-FITC (Vector FL1351), DAPI (Sigma-Aldrich D9542), ProLong Gold antifade reagent (Thermo Fischer P36930).
·*PB*: 0.1M phosphate buffer, pH 7.4.
·*PBS*: PB with 0.9% w/v NaCl.
·*Post-fixation solution*: 4% w/v formaldehyde in PBS.

Fluorescent staining of perineuronal nets. Before begining the staining, the samples, stored at -30°C (see *Brain dissection* in section 2.3), were first thawed at 4°C 30min and then tempered at RT 30min (this prevents the formation of ice crystals in the samples that are going to be re-stored at -30°C). The samples were incubated in cold post-fixation solution (-20°C) for 15min. They were allowed to air dry at RT for 20min and three washes of 15min were performed in PB. The samples were then incubated overnight with WFA-FITC 1:250 in PB with 0.25% v/v Triton X-100. The incubation was carried out in the dark, in a humid chamber, at 4°C and on a flat surface. The next day, three 15-minute washes were performed in PB. Each slice was incubated in 100µL of DAPI 1:1500 in PB at RT, in the dark, for 5min and two washes of 15min were performed in PB. They were covered with ProLong Gold and coverslips and left to dry 24h in the dark.

Fluorescence microscopy and analysis. The images were taken with an Olympus U-RFL-T fluorescence microscope (Olympus DP70 camera) located in the Center for Biomedical Technology (CTB, campus of Montegancedo of the Polytechnic University of Madrid), in the context of a collaboration with Dr. Javier de Felipe. Images were obtained at x10, x20 and x40 from different regions of the prefrontal cortex (from both hemispheres) to +3.7mm from Bregma approximately. Two x40 images were taken to encompass all layers in insular (IC), anterior cingulate (ACC), dorsal prelimbic (dPL), ventral prelimbic (vPL) and infralimbic cortex (IL), and one in ventral (vOFC) and lateral orbitofrontal cortex (lOFC), according to the atlas of Paxinos and Watson (2007) (Figure 14). They were laid out in Adobe Photoshop CC. The images were analysed with the free software Fiji (ImageJ). From each region, the number of perineuronal networks (PNN, perineuronal nets) per square micrometer (PNN/μm^2) was calculated, regardless of their intensity.

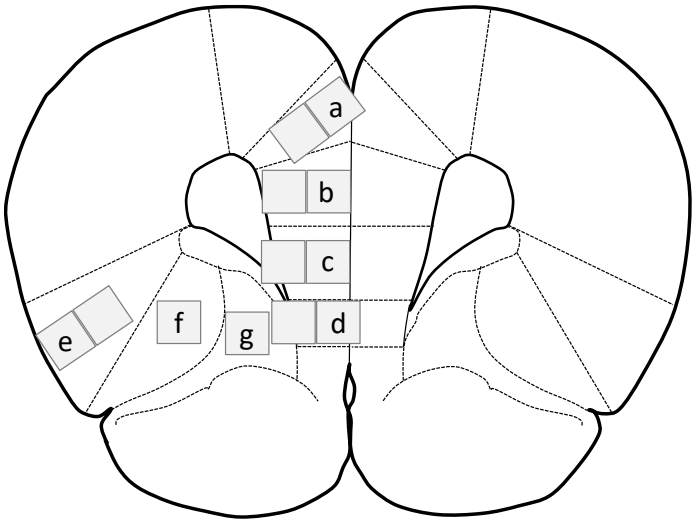


Figure 14. Location of the obtained micrographs:

a) anterior cingulate cortex
b) dorsal prelimbic cortex
c) ventral prelimbic cortex
d) infralimbic cortex
e) insular cortex
f) lateral orbitofrontal cortex
g) medial orbitofrontal cortex

Issues encountered during the analysis. We have encountered to types of problems during PNN analysis: some slices were deteriorated and there was a notable amount of variation in the staining from one brain to another (Figures 15 and 16). Some potential reasons for these issues are an incident with the freezers and the high amount of slices that had to be run in parallel. To deal with the problem of variance, two strategies were assayed: to normalize with respect to the total PNN of each subject or to perform ANCOVA using the total number of PNN of each subject as covariate. To solve the lack of certain data when calculating the total value per subject, we applied an imputation procedures based on bootstrapping (implemented in IBM Statistics). These imputated data were only considered for normalization purposes but were not included in the ANOVAs or ANCOVAs.

The ANCOVA approach had to be discarded as the covariate showed a significant interaction with the treatment and/or the time of abstinence. Therefore, the PNNs were analysed as a proportion of the total of each animal. Since we had five different imputation models for the lost values, the results are presented as ranges.

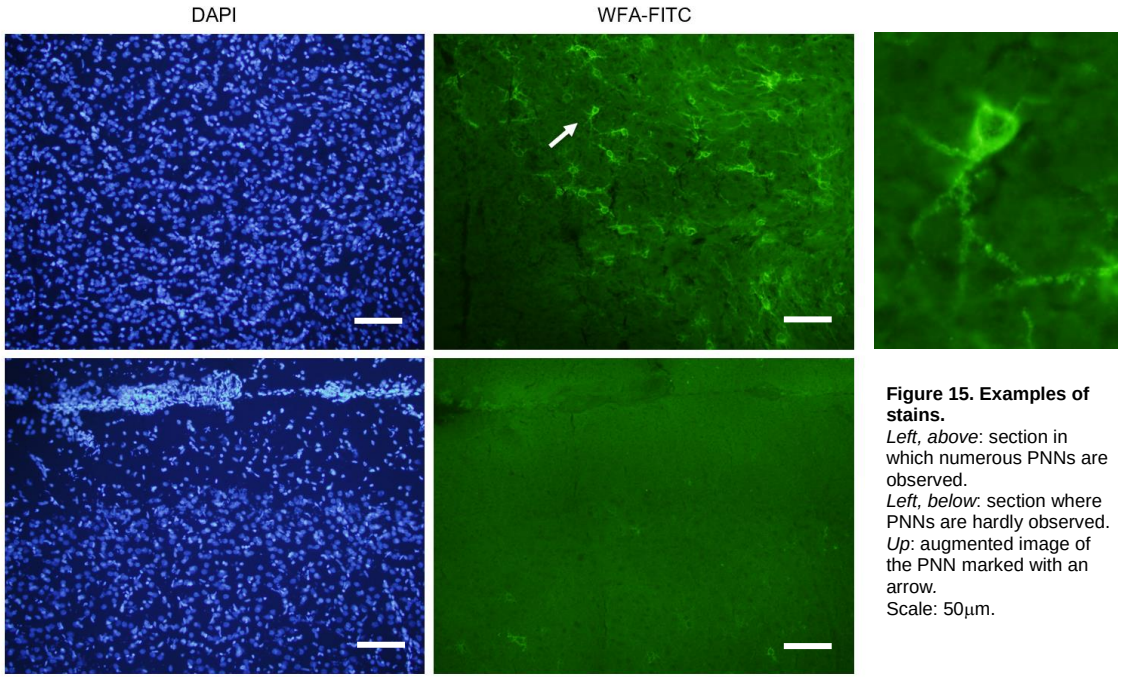


Figure 15. Examples of stains.
Left, *above*: section in which numerous PNNs are observed.
Left, *below*: section where PNNs are hardly observed.
Up: augmented image of the PNN marked with an arrow.
Scale: 50μm.

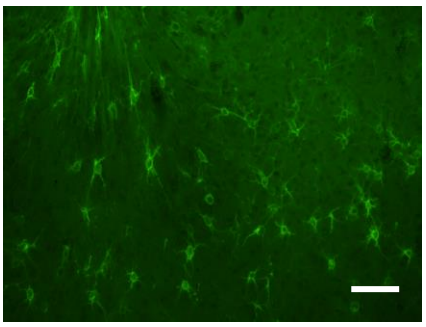


Figure 16. Example of a good stain.
Scale: 50μm.

2.10. STATISTICAL ANALYSIS

$$SE_{\bar{x}} = \frac{SD}{\sqrt{n}}$$

Karl Pearson, 1857-1936.

Data presentation. The values are presented in tables and/or graphs. When they are shown in tables, the mean and the standard deviation (SD) are presented to inform about the distribution of the sample. When they are shown in graphs, each individual value is depicted and the average and the standard error of the mean (SE) are presented, except in the number of lever pressures in the self-administration sessions, where the mean and the SE are shown.

Univariate statistics. To compare two groups, Student's test was performed in the case of equality of variances (paired or unpaired) and Welch's test in the case of inequality of variances (always unpaired). Two-tailed tests were performed except to analyse the incubation, because there is sufficient information in the literature to predict the direction of the effect. To analyse the evolution of a value throughout the sessions in a single group, one-way ANOVA was performed with repeated measurements. To compare groups subdivided into two levels (treatment, abstinence), two-way ANOVAs followed by *post hoc* tests was performed. When there was no variance homogeneity, we transformed the data into decimal logarithm, square root or inverse. ANCOVA was also performed to study the incubation of seeking, controlling the number of active lever presses with the number of inactive lever presses. The following effect sizes are presented: Cohen's *d* for t-test and η^2 for ANOVA and ANCOVA.

$$d = \frac{\bar{x}_t - \bar{x}_c}{s_{pooled}}$$

$$\eta^2 = \frac{SS_{Between}}{SS_{Total}}$$

Ronald Fisher, 1890-1962.

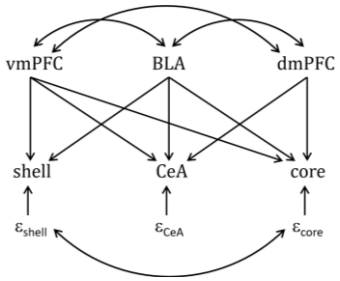
Bivariate statistics. To detect correlated variables that could have a psychobiological meaning (as behavioural predictors of incubation or biochemical variables that, although localized in different brain regions, were functionally related), a screen of Pearson and Spearman correlations (IBM Statistics) followed by their respective comparisons between groups were performed (Excel). The Fisher transformation was applied to the *r*, obtaining *r'*, and then the *Z* value and its *p* value were calculated to compare the correlations between groups. Two types of comparisons were made for each substance: a) the wd1 group versus its wd1 control and its wd30 group (for example, "cocaine wd1" versus "saline wd1" and versus "cocaine wd30"), and b) group wd30 versus its control wd30 and versus its group wd1 (following with the same example, "cocaine wd30" versus "saline wd30" and versus "cocaine wd1"). To select the correlations with differences between groups, no multiple comparisons correction was applied. We filtered them on the basis of three criteria: 1) a sample size ≥6 in the compared groups; 2) a significant correlation ($p<.05$) in the group to be tested ("cocaine wd1" or "cocaine wd30" in the examples); and 3) *Z* of both comparisons (versus "saline wd1" and "cocaine wd30" in the first example) with a *p*-value <.05.

$$z = \frac{r_1' - r_2'}{s} \quad \begin{cases} r' = \frac{1}{2}\ln\frac{1+r}{1-r} \\ s = \sqrt{\frac{1}{n_1 - 3} + \frac{1}{n_2 - 3}} \end{cases}$$

Principal component analysis (PCA). To reduce dimensions and find latent variables from behavioural data and from gene expression data, PCAs performed made using SPSS. The variables had to meet two criteria to be included in the analysis: to have commonality values ≥.600 and to be not correlated with more than two components. The analyses had to meet three criteria: a Kaiser-Meyer-Olkin (KMO) sample adequacy measure (MSA) ≥.600, a value of $p<.05$ in the Bartlett's sphericity test, and a non-zero determinant (at least >.00001). To avoid the presence of variables correlated with more than one component and to facilitate their interpretation, the orthogonal Varimax rotation was carried out if the components were not correlated (it was fulfilled in all cases). The number of components was chosen according to three criteria: an eigenvalue greater than unity, an accumulated variance >60%, and the shape of the sedimentation graph. No component correlated with only one variable was accepted. To interpret the meaning of the components, we looked at the variables with which they correlated significantly (Pearson's correlation coefficient), applying the correction suggested by Bonferroni for multiple comparisons: those correlations with a value of $p<.05/x$ are significant, where x is the number of correlations studied. The values of each animal for each component were obtained from IBM Statistics program in order to perform univariate and bivariate analysis with them (Field, 2013).

Structural equation modelling (SEM). As a multivariate method, SEM allows us to study the relationship between several variables at once, being possible to observe differences in this relationship even though no differences are observed in univariate analyses (Poirier *et al*., 2008). We perform path analysis with maximum likelihood estimation (MLE) in the SPSS Amos 22 program (IBM). The variable introduced was the PSD95/gephyrin ratio in each animal in each of the six regions studied, as a measure of synaptic changes in glutamatergic and GABAergic transmission. The model consisted of direct connections known bibliographically from glutamatergic regions (BLA, dmPFC, vmPFC) to GABAergic regions (nucleus accumbens core and shell, and CeA). Specifically, connections from the anterior cingulate and dorsal prelimbic cortex (both in dmPFC) and from the ventral prelimbic cortex (in vmPFC) to the core region of the nucleus accumbens; from vmPFC to the shell region of the nucleus accumbens (Voorn *et al*., 2004); from the basolateral amygdala to both regions of the nucleus accumbens and the central nucleus of the amygdala; and connections from both medial regions of the prefrontal cortex to CeA (Pape and Pare, 2010). It was also composed of covariances (CV) between glutamatergic regions (dmPFC↔vmPFC, dmPFC↔BLA, vmPFC↔BLA), and CV between GABAergic regions (core↔shell). These CVs denote not only possible bidirectional connections, but also possible dissection errors between contiguous regions (dmPFC/vmPFC, core/shell). In addition, working with CV allows us to avoid bidirectional connections that would otherwise generate less accurate models given the low sample size (Boucard *et al.*, 2007).

Using anatomical connections when performing SEM, the comparisons between groups are accurate even when the goodness-of-fit estimators of the models are not, at least when using functional data (Protzner and McIntosh, 2006). Even so, three goodness-of-fit estimators were calculated (Kinnavane *et al*., 2014). First χ^2 which, although not accurate for low sample sizes, allows us to make comparisons between groups (see below). In addition, their associated value of p (recommended >.05) and their degrees of freedom (df, recommended ratio $\chi^2/df<2$) were presented. Two other more appropriate estimators for low sample sizes were also calculated: the comparative fit index (CFI), recommended >.9, and the root mean square error of approximation (RMSEA), recommended <.8. In addition, to validate the model, these same parameters of goodness of fit were calculated using higher sample sizes, obtained with different combinations of the experimental groups, since the original groups have low sample sizes (n=7-9). We presented the squares of the multiple correlation coefficients of the endogenous variables and the standardized regression weights (SRW) of each path (with their associated p values and their standard error), as well as the p-values associated with the CVs and the value of the correlations. To compare the SRW between the different experimental groups, the $\Delta\chi^2$ method was used. This method is based on the comparison of two models: the original model and one in which the equality of a path between two experimental groups is forced (for example, the equality in SWR is forced between the heroin wd30 and saline wd30 groups for the BLA>shell path), one by one. We calculate the value of χ^2 for each model, its difference ($\Delta\chi^2$) and the associated p, with a degree of freedom of 1, since the comparisons are performed path by path (Δdf=1). The comparisons in which p<.05 are obtained are considered to be significantly different.

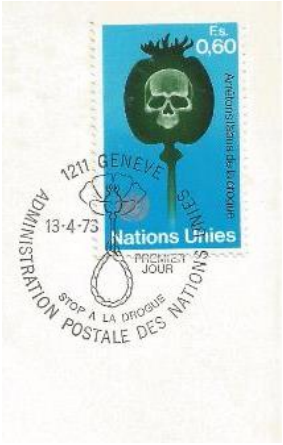


Model used in the study.

External variables:
- PSD95/gephyrin ratio of vmPFC, dmPFC and BLA;
- errors of shell, core and CeA (ε).

Internal variables:
- PSD95/gefirina ratios of shell, core and CeA.

One-headed arrows: regressions.
Two-headed arrows: covariances.



3. RESULTS

1st Goal: SELF-ADMINISTRATION AND SEEKING INCUBATION

3.1. SUBSTANCE SELF-ADMINISTRATION AND SEEKING INCUBATION

Incubation of seeking. As expected, incubation of seeking was observed for the three substances studied (*Experiment 1*) in the Lewis rat strain (Table 6, Figure 17D, E, F), as analysed by *t*-test as well as by ANCOVA, controlling the number of active lever presses with the number of inactive lever presses.

Table 6. Incubation test statistics for cocaine, heroin and sucrose by *t*-test and by ANCOVA.

Analysis	Cocaine	Heroin	Sucrose
t-test active lever,	*t*(7)=-3.27, *d*=2.47,	*t*(8.022)=-2.669, *d*=1.89,	*t*(13)=-2.483, *d*=1.38,
1 tail:	*p*=.0068**	*p*=.0142*	*p*=.0137*
2 tails:	*p*=.014*	*p*=.028*	*p*=.027*
t-test inactive lever,	*t*(7)=-1.581,	*t*(14)=-0.460,	*t*(13)=-1.361,
2 tails:	*p*=.158	*p*=.653	*p*=.197
ANCOVA active lever, controlled by inactive lever:	$F(1,6)=6.024$, $\eta^2=.390$, *p*=.049*	$F(1,13)=6.372$, $\eta^2=.304$, *p*=.025*	$F(1,12)=6.329$, $\eta^2=.344$, *p*=.027*

Self-administration behaviour. Then a second experiment was performed to obtain the tissue samples (*Experiment 2*). The rats acquired the lever-press behaviour with the three substances (Figure 17G, H, I) as in the first experiment (Figure 17A, B, C). As the sessions went on, the rats learned the behaviour and self-administered more cocaine, heroin or sucrose (linear contrast with *p*<.0001 for the three substances). In addition there were no differences, for each substance, in the behaviour pattern of rats from *Experiments 1* (test) and *2* (tissue) nor between rats going through a day (wd1) or thirty days (wd30) of forced abstinence, nor was there any interaction between these factors (Table 7). The rats in the control groups (saline and water) barely pressed the lever throughout the sessions (Figure 17).

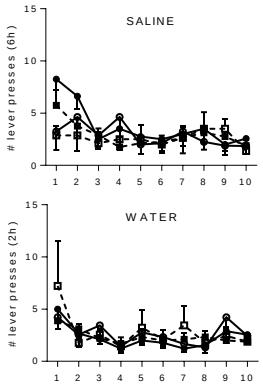


Figure 17. Lever-press behaviour of control groups. Legend in Figure 18.

Given that the pattern of consumption between experiments was indistinguishable, we assumed that the incubation of the seeking observed in *Experiment 1* would be evident also in the animals of *Experiment 2* if we subjected them to the respective extinction tests on days 1 or 30 of abstinence. As a conclusion, the tissue samples obtained are consider to be representative of animals undergoing incubation of seeking.

Table 7. Statistics of self-administration sessions of cocaine, heroin and sucrose by repeated measures factorial ANOVA (session factor) with lever (active/inactive), withdrawal time (wd1/wd30) and experiment (1/2) factors.

Effect in ANOVA	Cocaine	Heroin	Sucrose
lever	***F*(1,22)=66.56, *p*=.000, $\eta^2=.410$**	***F*(1,28)=26.51, *p*=.000, $\eta^2=.151$**	***F*(1,29)=168.19, *p*=.000, $\eta^2=.457$**
*lever*wd*	*F*(1,22)=0.00, *p*=.949	*F*(1,28)=1.21, *p*=.281	*F*(1,29)=0.07, *p*=.790
*lever*exp*	*F*(1,22)=0.05, *p*=.826	*F*(1,28)=0.59, *p*=.447	*F*(1,29)=0.01, *p*=.931
*lever*wd*exp*	*F*(1,22)=0.39, *p*=.536	*F*(1,28)=1.09, *p*=.306	*F*(1,29)=0.01, *p*=.941
session	***F*(4.7,103.8)=8.27, *p*=.000, $\eta^2=.062$**	***F*(2.6,72.7)=5.81, *p*=.002, $\eta^2=.059$**	***F*(4.8,139.3)=42.45, *p*=.000, $\eta^2=.128$**
*session*wd*	*F*(4.7,103.8)=1.00, *p*=.418	*F*(2.6,72.7)=0.55, *p*=.623	*F*(4.8,139.3)=1.56, *p*=.178
*session*exp*	*F*(4.7,103.8)=0.56, *p*=.722	*F*(2.6,72.7)=0.34, *p*=.767	*F*(4.8,139.3)=0.54, *p*=.735
*session*wd*exp*	*F*(4.7,103.8)=0.44, *p*=.807	*F*(2.6,72.7)=1.25, *p*=.298	*F*(4.8,139.3)=0.32, *p*=.894
lever*session	***F*(3.8,83.4)=12.08, *p*=.000, $\eta^2=.070$**	***F*(2.4,67.8)=3.48, *p*=.028, $\eta^2=.031$**	***F*(4.7,137.7)=63.79, *p*=.000, $\eta^2=.163$**
*lever*session*wd*	*F*(3.8,83.4)=0.99, *p*=.413	*F*(2.4,67.8)=1.00, *p*=.384	*F*(4.7,137.7)=1.00, *p*=.420
*lever*session*exp*	*F*(3.8,83.4)=0.40, *p*=.797	*F*(2.4,67.8)=1.11, *p*=.345	*F*(4.7,137.7)=0.23, *p*=.944
*lever*session*wd*exp*	*F*(3.8,83.4)=0.93, *p*=.450	*F*(2.4,67.8)=0.53, *p*=.624	*F*(4.7,137.7)=0.59, *p*=.697
wd	*F*(1,22)=0.75, *p*=.395	*F*(1,28)=0.48, *p*=.493	*F*(1,29)=0.22, *p*=.639
exp	*F*(1,22)=0.57, *p*=.457	*F*(1,28)=0.08, *p*=.782	*F*(1,29)=0.12, *p*=.731
*wd*exp*	*F*(1,22)=0.75, *p*=.394	*F*(1,28)=1.15, *p*=.292	*F*(1,29)=0.12, *p*=.736

Behavioural predictive variables of seeking incubation. We did not find any behavioural parameter during the self-administration sessions of *Experiment 1* that correlated with the degree of incubation. Therefore, in the rats of a month of abstinence from *Experiment 2*, correlations between behavioural and biochemical parameters will not be studied.

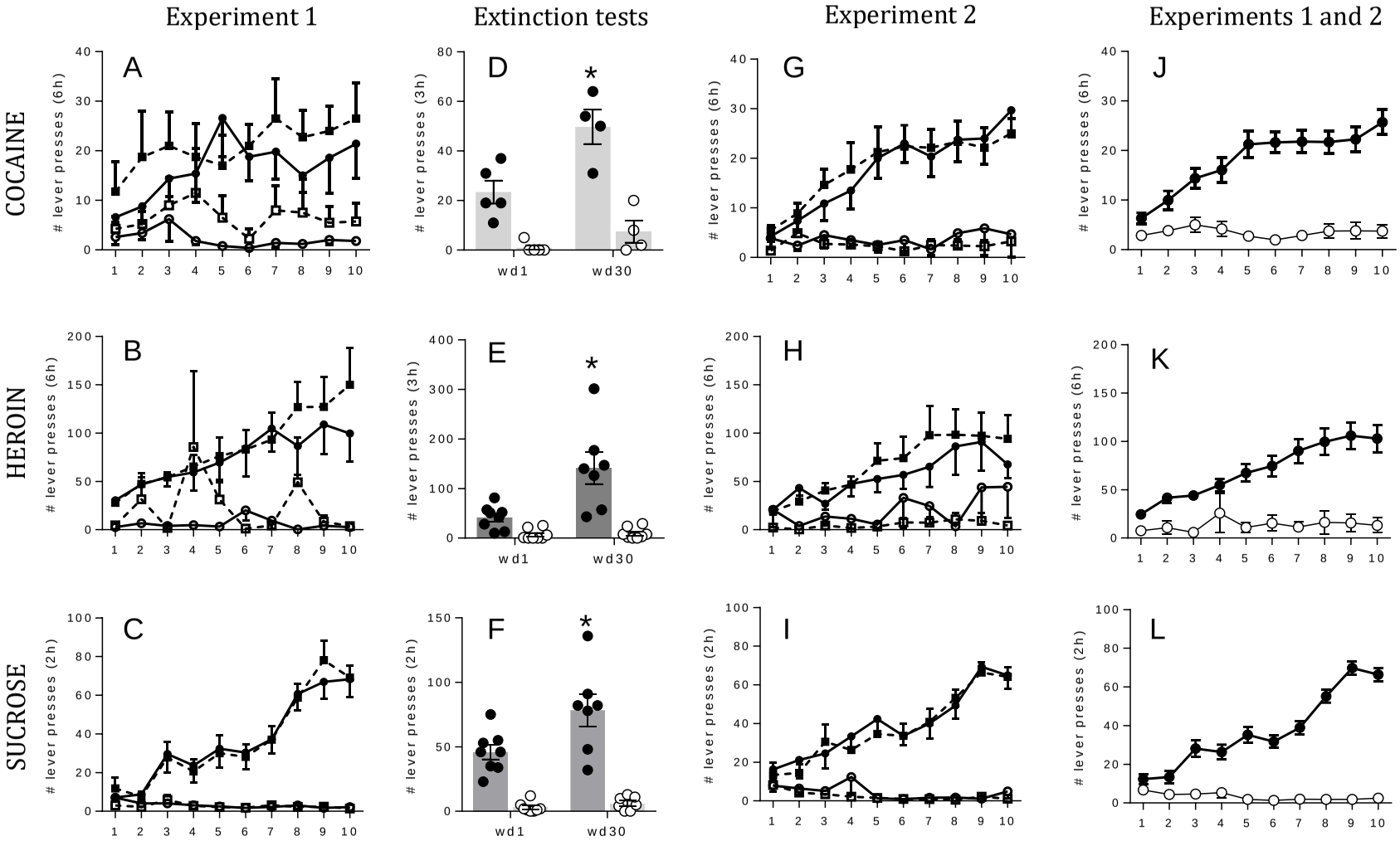


Figure 18. Sessions of self-administration and extinction test. Number of active (*black symbols*) and inactive (*white symbols*) lever presses during the self-administration sessions of cocaine (A, G, J), heroin (B, H, K) and sucrose (C, I, L) in the *Experiment 1* (A, B, C), *2* (G, H, I) or both (J, K, L). In the figures with the disaggregated experiments, the animals of the groups of one day of abstinence (*circles, continuous lines*) and one month of abstinence (*squares, dashed lines*) are shown separately. Lever presses are also presented during the extinction tests for cocaine (D), heroin (C) and sucrose (F). Mean±SE.

2nd Goal: CHANGES IN GLUTAMATERGIC AND GABAERGIC PARAMETERS DURING SEEKING INCUBATION

3.2. SYNAPTIC SCAFOLDING PROTEINS LEVELS

Levels of PSD95 and gephyrin by brain region. The levels of both PSD95 and gephyrin, the main postsynaptic scaffolding proteins of glutamatergic and GABAergic receptors, remained stable despite treatments and withdrawal times. The only change observed was the increase in core of the PSD95/gephyrin ratio both at short times and at long times of sucrose withdrawal (Figure 19).

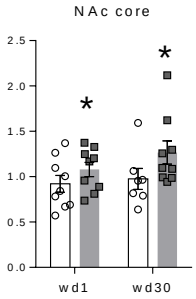


Figure 19. PSD95/gephyrin ratio in NAc core in experiment WZ. *White*: water; *grey*: sucrose. $F(1,30)=4.562$, $p=.041$, $\eta^2=.128$.

Synaptic coherence between brain regions by SEM. Subsequently, we studied the degree of synaptic coherence between regions through the correlations between the PSD95/gephyrin ratio, the main postsynaptic proteins of the excitatory and inhibitory synapses. We chose this ratio as an index of net excitatory activity (Keith, 2008; Yu and Blas, 2008). First, we validated the model by feeding it with compacted groups, thus obtaining larger sample sizes (the composition of these is presented in Figure 20). The goodness-of-fit estimators are presented in Table 8. We then calculated the value of the parameters for each experimental group (Table 9 and Figure 21 for compacted groups and Table 10 and Figure 22 for individual groups).

Table 8. Goodness-of-fit estimators of the different grouping models for the same path design.

	χ^2	df	*p*	χ^2/df	CFI	RMSEA
Compacted groups:						
model A	1.473	3	.689	0.491	1	0
model B	4.814	6	.568	0.802	1	0
model C	10.908	12	.537	0.909	1	0
Original groups:						
experiment SCH	46.392	18	.000	2.577	.739	.201
experiment WZ	23.358	12	.025	1.946	.595	.178

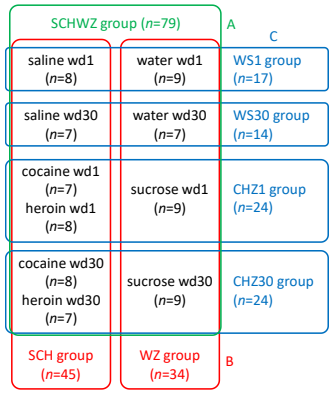


Figure 20. Composition of the compacted groups in SEM. In model A there is only one group, formed by all the individuals. In model B the individuals of each experiment constitute one group. In model C, individuals are divided according to whether they are controls or treated groups and depending on the time of abstinence.

It is noteworthy that the values of the squared multiple correlations (SMC), which give an idea of the capacity of the model to explain the variance of, in our case, the values of core, shell and CeA, are on average higher for CeA than for core or, above all, shell, and more in the cocaine and heroin groups than in the sucrose groups. This could be indicating that the selected glutamatergic regions, and with the chosen variable (PSD95/gephyrin), give a good account of the functional relationships of CeA but not so much of the functional relationships of the nucleus accumbens.

Group comparisons. As we were not interested in the numerical value of each parameter, but in the differences between groups, we compared each of the paths using the $\Delta\chi^2$ method. The results are presented in Table 11 and Figure 24, and a summary thereof in Figure 23. The most striking result was that the incubation of seeking of the three substances exhibited changes in the way in which the glutamatergic regions were related to the central nucleus of the amygdala (CeA). On the one hand, a qualitative change was observed in the relationship between the basolateral amygdala (BLA) and CeA after the incubation of cocaine seeking, from negative to positive (Figure 24I). On the other hand, a quantitative change was observed in the relationship between dmPFC and CeA, as this relationship disappeared after one month of sucrose withdrawal (Figure 24G). In the heroin abstinent rats both changes were observed (Figure 24I, G). The relationship with vmPFC was mixed in the rats of cocaine and heroin, strengthening in the first case and weakening in the second (Figure 24H).

On the contrary, we did not observe any common change between substances centered in any of the two studied regions of the nucleus accumbens, although there were specific changes of substance. An intensification of the vmPFC>core pathway was found after one month of heroin withdrawal (Figure 24E) and a weakening of the dmPFC>core pathway after one month of sucrose abstinence (Figure 24D).

Finally, we observed changes apparently due to the effect of cocaine and heroin use (and not delayed abstinence) in the dmPFC>core pathway (Figure 24D) and BLA>shell pathway (Figure 24C), respectively.

Table 9. SEM parameters for the compacted groups.

	model A		model B				model C							
	SCHWZ (79)		SCH (45)		WZ (34)		WS1 (17)		CHZ1 (24)		WS30 (14)		CHZ30 (24)	
Standardized Regression Weights:	SRW	*p*	SRW	*p*	SRW	*p*	SRW	*p*	SRW	*p*	SRW	*p*	SRW	*p*
vmPFC→shell	0.060	*0.571*	0.058	*0.675*	0.068	*0.695*	-0.203	*0.421*	0.275	*0.185*	-0.109	*0.690*	0.054	*0.769*
vmPFC→CeA	**0.332****	*0.001*	**0.445****	*<0.001*	0.017	*0.918*	0.185	*0.452*	**0.444***	*0.023*	0.129	*0.596*	**0.378***	*0.016*
vmPFC→core	0.006	*0.959*	-0.078	*0.601*	0.194	*0.247*	0.144	*0.583*	-0.029	*0.885*	0.063	*0.776*	0.001	*0.994*
BLA→shell	**0.341****	*0.001*	**0.404****	*0.004*	0.068	*0.697*	-0.123	*0.626*	-0.01	*0.962*	0.045	*0.871*	**0.475***	*0.010*
BLA→CeA	0.197	*0.061*	**0.373****	*0.004*	-0.145	*0.386*	-0.19	*0.426*	-0.138	*0.477*	-0.271	*0.282*	**0.501****	*0.003*
BLA→core	-0.012	*0.919*	0.020	*0.900*	-0.103	*0.542*	-0.035	*0.891*	-0.015	*0.939*	**-0.541***	*0.015*	0.155	*0.486*
dmPFC→CeA	0.107	*0.308*	-0.044	*0.732*	0.273	*0.103*	0.243	*0.307*	0.071	*0.708*	-0.28	*0.268*	0.008	*0.962*
dmPFC→core	**0.234***	*0.037*	0.231	*0.139*	0.172	*0.308*	-0.067	*0.750*	**0.389***	*0.043*	**0.454****	*0.002*	0.116	*0.599*
Correlations:	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*
BLA↔vmPFC	0.123	*0.282*	0.135	*0.374*	0.088	*0.616*	-0.26	*0.313*	0.239	*0.268*	-0.11	*0.690*	0.160	*0.452*
BLA↔dmPFC	0.194	*0.092*	**0.338***	*0.034*	-0.171	*0.333*	0.076	*0.762*	-0.056	*0.791*	0.281	*0.324*	0.360	*0.106*
vmPFC↔dmPFC	-0.016	*0.888*	-0.073	*0.628*	0.100	*0.567*	0.246	*0.337*	-0.11	*0.603*	-0.119	*0.666*	-0.057	*0.787*
ε_{shell}↔ε_{core}	0.000	*0.998*	0.023	*0.877*	-0.107	*0.541*	**0.569***	*0.047*	-0.134	*0.528*	**-0.763***	*0.027*	-0.036	*0.864*
Squared Multiple Correlations:	SMC		SMC		SMC		SMC		SMC		SMC		SMC	
core	0.054		0.065		0.087		0.025		0.155		0.366		0.050	
CeA	0.184		0.375		0.110		0.162		0.186		0.227		0.457	
shell	0.125		0.173		0.010		0.043		0.074		0.015		0.237	

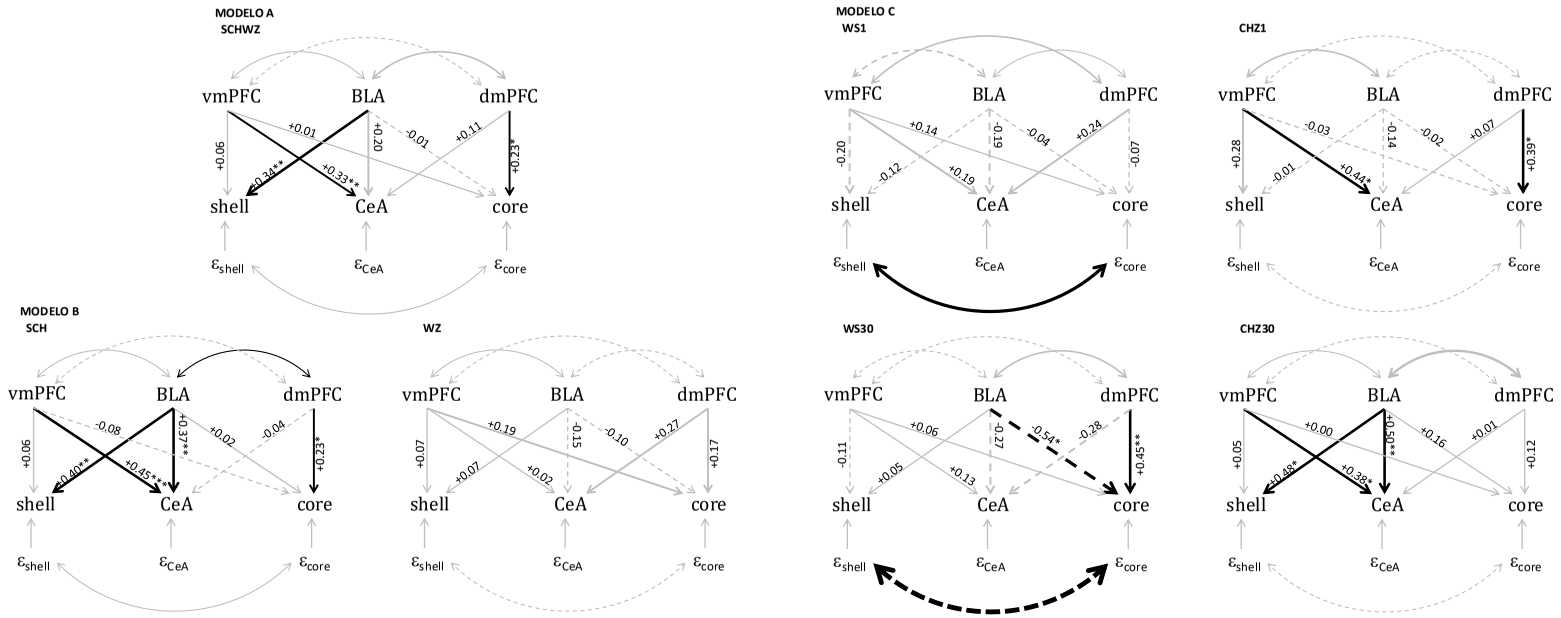


Figure 21. Diagrams of the compacted groups. The structural equation models for the compacted groups are presented. *One-headed arrows*: regressions; *two-headed arrows*: covariances; *continuous lines*: positive values; *dashed lines*: negative values; *grey lines*: non-significant parameter; *black lines*: significant parameter.

Table 10. SEM parameters of the individual groups.

Standardized Regression Weights:	saline wd1 (8) SRW	*p*	cocaine wd1 (7) SRW	*p*	heroin wd1 (8) SRW	*p*	saline wd30 (7) SRW	*p*	cocaine wd30 (8) SRW	*p*	heroin wd30 (7) SRW	*p*	water wd1 (9) SRW	*p*	sucrose wd1 (9) SRW	*p*	water wd1 (7) SRW	*p*	sucrose wd30 (9) SRW	*p*
vmPFC→shell	-0.052	*.895*	-0.013	*.974*	0.494	*.141*	**-0.762***	*.012*	-0.172	*.440*	0.071	*.862*	**-0.693***	*.013*	-0.18	*.633*	0.324	*.416*	0.245	*.486*
vmPFC→CeA	0.478	*.157*	**1.425****	*<.001*	-0.049	*.829*	0.089	*.835*	**0.922****	*.002*	**0.528****	*.007*	0.143	*.677*	0.422	*.194*	0.015	*.963*	-0.365	*.385*
vmPFC→core	0.771	*.061*	**-1.034***	*.019*	0.232	*.555*	**0.620****	*.002*	-0.328	*.536*	**-0.9*****	*<.001*	-0.19	*.627*	**0.723****	*<.001*	0.005	*.987*	0.634	*.152*
BLA→shell	-0.064	*.873*	0.516	*.193*	0.036	*.915*	-0.328	*.280*	**0.778****	*<.001*	0.311	*.448*	-0.291	*.301*	0.257	*.495*	0.105	*.792*	-0.025	*.943*
BLA→CeA	-0.479	*.123*	**-0.99****	*.008*	**-0.93****	*.001*	-0.155	*.720*	0.419*	*.030*	**0.776****	*<.001*	0.473	*.193*	-0.48	*.143*	-0.271	*.410*	-0.178	*.570*
BLA→core	0.421	*.275*	0.554	*.221*	0.315	*.518*	-0.337	*.090*	0.320	*.368*	**0.291****	*.007*	-0.222	*.587*	0.017	*.918*	**-0.636***	*.031*	-0.181	*.583*
dmPFC→CeA	0.050	*.884*	**0.789***	*.029*	**-1.2*****	*<.001*	-0.109	*.789*	0.139	*.640*	-0.242	*.297*	0.625	*.078*	0.185	*.537*	-0.593	*.066*	0.631	*.125*
dmPFC→core	-0.612	*.096*	-0.210	*.628*	0.695	*.153*	**0.403****	*.002*	-0.312	*.548*	**0.966****	*<.001*	-0.03	*.932*	**0.495****	*.001*	**0.618****	*<.001*	-0.505	*.245*
Correlations:	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*	R	*p*
BLA↔vmPFC	-0.296	*.454*	0.471	*.294*	-0.165	*.667*	-0.356	*.409*	-0.091	*.812*	0.366	*.397*	-0.317	*.394*	0.406	*.289*	0.346	*.417*	0.195	*.590*
BLA↔dmPFC	0.340	*.397*	0.439	*.322*	-0.654	*.149*	0.190	*.646*	-0.118	*.758*	0.623	*.193*	-0.403	*.292*	-0.157	*.661*	0.285	*.496*	-0.034	*.923*
vmPFC↔dmPFC	0.503	*.237*	-0.395	*.366*	-0.294	*.458*	0.078	*.849*	-0.763	*.110*	0.599	*.206*	-0.236	*.517*	0.068	*.849*	-0.287	*.494*	0.667	*.118*
ε_{shell}↔ε_{core}	0.589	*.181*	-0.087	*.830*	-0.369	*.362*	-0.724	*.149*	0.339	*.398*	-0.838	*.114*	0.536	*.183*	-0.149	*.677*	-0.83	*.112*	-0.03	*.934*
Squared Multiple Correlations:	SMC		SMC		SMC		SMC		SMC		SMC		SMC		SMC		SMC		SMC	
core	.304		.606		.230		.796		.194		.945		.052		.824		.559		.212	
CeA	.604		.730		.756		.059		.766		.852		.311		.317		.519		.289	
shell	.005		.260		.239		.510		.658		.118		.437		.061		.139		.058	

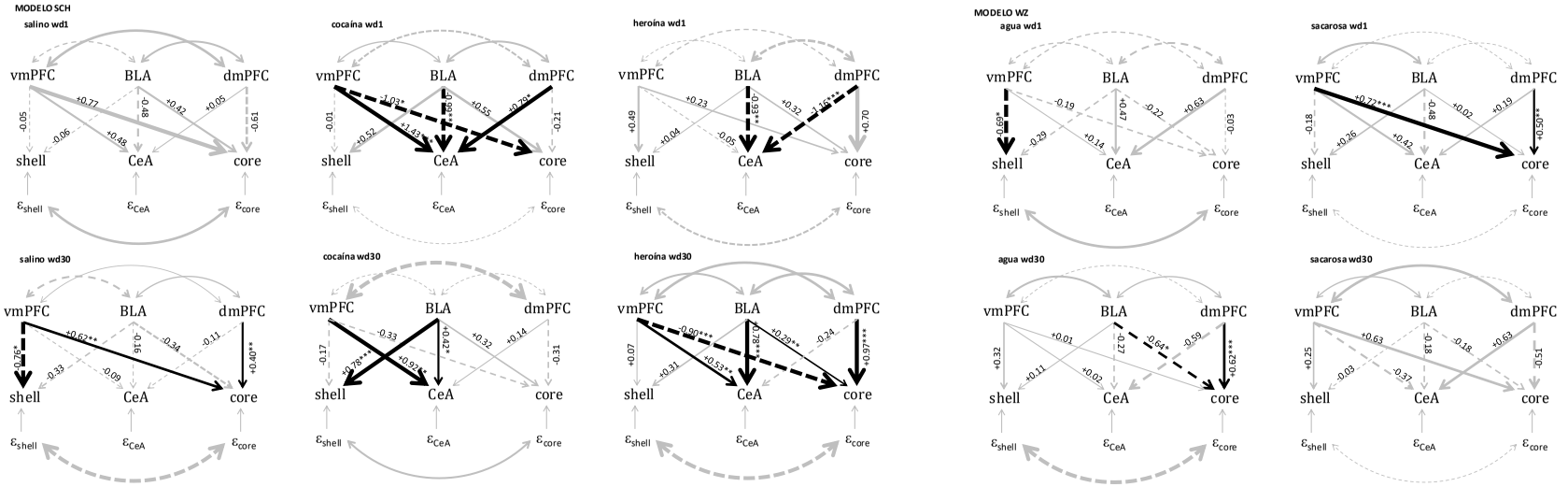


Figure 22. Diagrams of the individual groups. The structural equation models for the individual groups are presented. *One-headed arrows*: regressions; *two-headed arrows*: covariances; *continuous lines*: positive values; *dashed lines*: negative values; *grey lines*: non-significant parameter; *black lines*: significant parameter.

Table 11. Values of $\Delta\chi^2$ for each group comparison, path by path. The associated *p*-values are presented in parentheses.

		from vmPFC to						from BLA to						from dmPFC to			
		shell		CeA		core		shell		CeA		core		CeA		core	
saline wd1 *vs* saline wd30	$\Delta\chi^2$	0.650	*(.420)*	0.756	*(.385)*	1.063	*(.303)*	0.440	*(.507)*	0.040	*(.841)*	2.678	*(.102)*	0.091	*(.763)*	**3.689t**	*(.055)*
saline wd1 *vs* cocaine wd1	$\Delta\chi^2$	0.011	*(.916)*	**4.874***	*(.027)*	1.763	*(.184)*	0.529	*(.467)*	2.386	*(.122)*	0.228	*(.633)*	1.995	*(.158)*	0.008	*(.929)*
saline wd1 *vs* heroin wd1	$\Delta\chi^2$	0.873	*(.350)*	1.509	*(.219)*	0.171	*(.679)*	0.037	*(.847)*	1.507	*(.220)*	0.027	*(.869)*	**6.82***	*(.009)*	2.982	*(.084)*
cocaine wd1 *vs* cocaine wd30	$\Delta\chi^2$	0.072	*(.788)*	3.407	*(.065)*	1.078	*(.299)*	1.042	*(.307)*	**7.140***	*(.008)*	0.220	*(.639)*	1.375	*(.241)*	0.005	*(.944)*
saline wd30 *vs* cocaine wd30	$\Delta\chi^2$	3.068	*(.080)*	3.035	*(.081)*	1.715	*(.190)*	**3.745t**	*(.053)*	1.267	*(.260)*	2.152	*(.142)*	0.247	*(.619)*	1.274	*(.259)*
saline wd30 *vs* heroin wd30	$\Delta\chi^2$	0.145	*(.703)*	**4.249***	*(.039)*	**22.53***	*(.000)*	1.495	*(.221)*	1.400	*(.237)*	3.459	*(.063)*	0.220	*(.639)*	**9.97***	*(.002)*
heroin wd1 *vs* heroin wd30	$\Delta\chi^2$	0.002	*(.964)*	**4.792***	*(.029)*	**6.140***	*(.013)*	0.375	*(.540)*	**11.62***	*(.001)*	0.214	*(.644)*	3.268	*(.071)*	0.267	*(.605)*
water wd1 *vs* sucrose wd1	$\Delta\chi^2$	1434	*(.231)*	0.365	*(.546)*	2.869	*(.090)*	1.420	*(.233)*	3.280	*(.070)*	0.291	*(.590)*	1.264	*(.261)*	0.701	*(.402)*
water wd1 *vs* water wd30	$\Delta\chi^2$	2521	*(.112)*	0.125	*(.724)*	0.162	*(.687)*	0.319	*(.572)*	2.171	*(.141)*	1.024	*(.312)*	**5.104***	*(.024)*	**3.677t**	*(.055)*
water wd30 *vs* sucrose wd30	$\Delta\chi^2$	0.056	*(.813)*	0.629	*(.428)*	1.379	*(.240)*	0.049	*(.825)*	0.054	*(.816)*	0.167	*(.683)*	**3.972***	*(.046)*	**3.839t**	*(.050)*
sucrose wd1 *vs* sucrose wd30	$\Delta\chi^2$	0.696	*(.404)*	2.168	*(.141)*	0.045	*(.832)*	0.056	*(.813)*	0.013	*(.909)*	0.304	*(.581)*	1.226	*(.268)*	1.953	*(.162)*

t $p<.056$, *$p<.050$

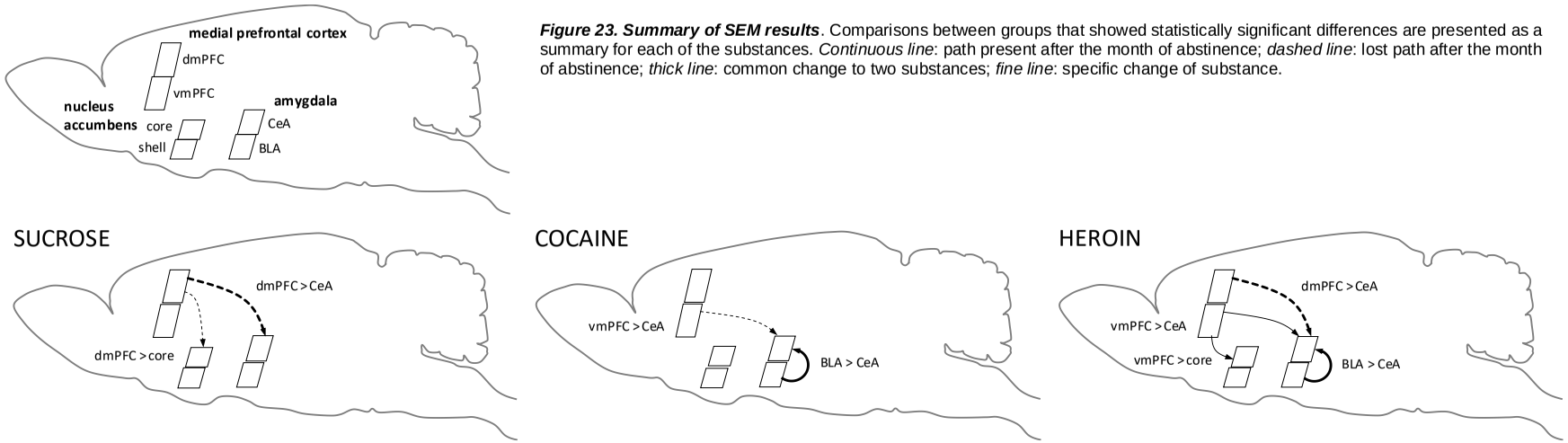


Figure 23. Summary of SEM results. Comparisons between groups that showed statistically significant differences are presented as a summary for each of the substances. *Continuous line*: path present after the month of abstinence; *dashed line*: lost path after the month of abstinence; *thick line*: common change to two substances; *fine line*: specific change of substance.

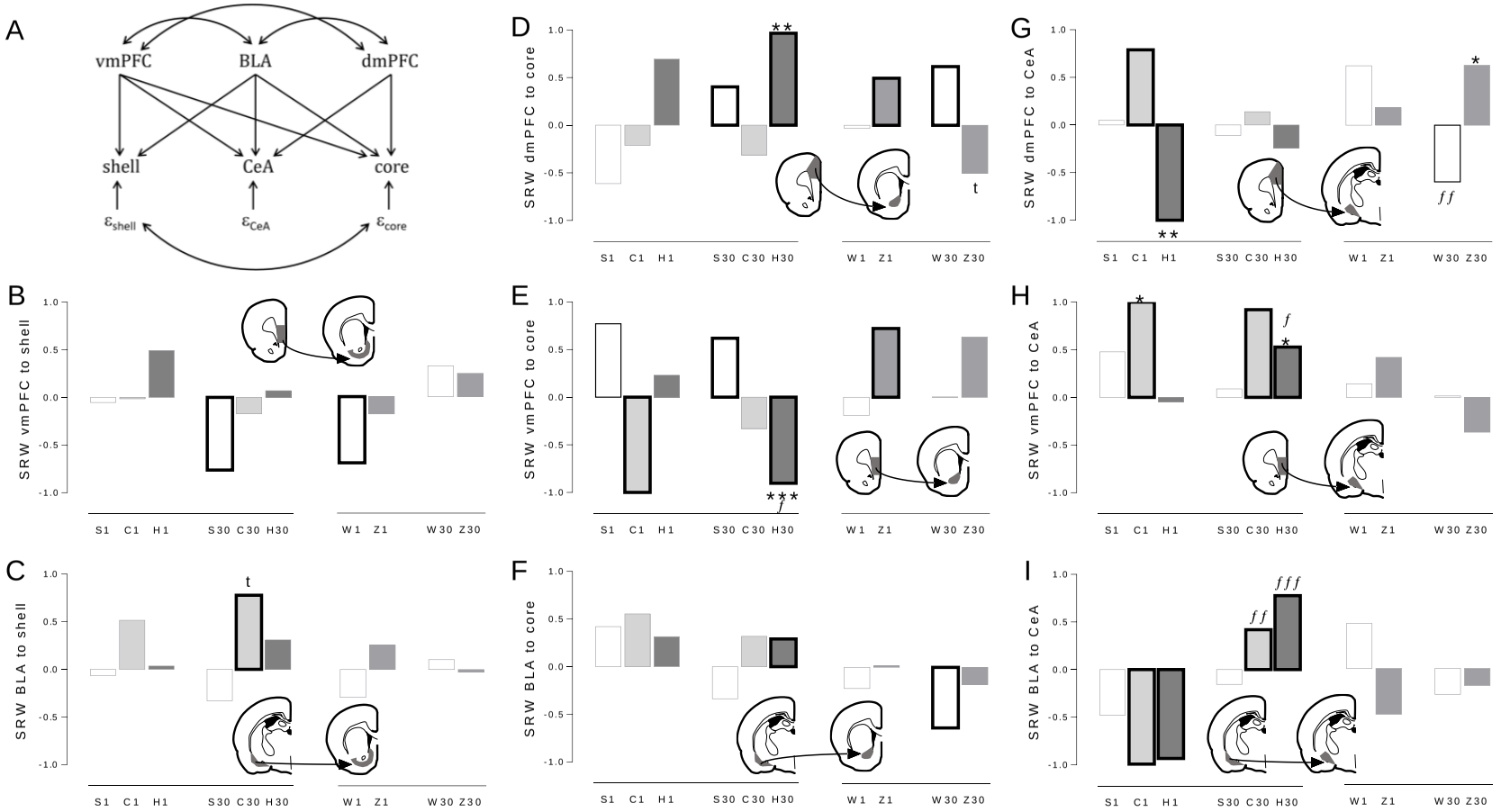


Figure 24. Group comparisons in SEM. A: Used model. B-I: Values of the regressions (SRW) for each path and group, and differences between groups. B-C: paths to shell; D-F: paths to core; G-I: path to CeA; D, G: paths from dmPFC; B, E, H: paths from vmPFC; C, F, I: paths from BLA; S: saline; C: cocaine; H: heroin; W: water; Z: sucrose; 1: wd1 group; 30: wd30 group. Outline of the histograms: *grey and thin*, SRW $p>.07$; *black and thin*, SRW $p<.07$; *black and thick*, SRW $p<.05$. Differences with the control group on the same day of abstinence: t $p<.054$, *$p<.05$, **$p<.01$, ***$p<.001$. Differences with the same treatment on different days of abstinence: *f* $p<.05$, *ff* $p<.01$, *fff* $p<.001$.

3.3. BRAIN AMINES LEVELS

Levels of amines in the control groups. We obtained similar levels of the different amines studied in the control groups of the experiments with water or saline as a vehicle. The slight differences may be due to the fact that the animals belong to different batches (although all the groups are composed of rats of at least three batches), to differences in the route of administration, to which some animals are operated and others are not, or to slight differences in age. The data are presented in Table 12 (mean±SD).

Table 12. Levels of brain amines (mean±SD) of control wd1 groups.

saline wd1	BLA	CeA	dmPFC	vmPFC	core	shell
GABA	20.2±2.9	30.0±4.8	12.0±1.8	14.0±1.5	24.7±3.8	28.2±5.2
L-glutamate	44.6±4.3	34.3±1.6	46.7±8.4	46.9±4.2	35.1±4.7	31.2±5.3
L-aspartate	17.1±2.5	19.5±1.2	29.1±4.7	14.1±1.6	18.3±2.8	18.3±2.9
taurine	23.6±5.4	22.7±2.6	22.5±6.3	30.2±3.7	32.4±3.4	27.3±5.2
glycine	5.2±2.0	6.5±1.4	6.9±1.0	4.4±2.7	6.2±1.6	10.3±2.9
L-glutamine	23.1±2.5	23.5±1.2	11.0±7.2	21.5±5.7	29.8±3.3	26.7±6.8
D-serine	0.8±0.2	0.2±0.1	0.7±0.2	3.5±0.7	0.7±0.1	0.6±0.1
L-serine	5.4±0.5	5.6±0.4	4.8±0.9	7.2±1.3	4.4±0.6	3.4±0.7

water wd1	BLA	CeA	dmPFC	vmPFC	core	shell
GABA	22.0±2.4	19.6±5.4	10.5±2.3	12.7±3.0	18.2±3.8	23.5±5.9
L-glutamate	41.8±7.0	29.8±8.1	40.0±7.5	35.2±2.6	32.0±4.6	25.3±3.9
L-aspartate	17.4±2.4	15.3±4.0	11.0±2.3	13.6±1.3	17.5±2.4	11.1±2.3
taurine	25.9±4.5	18.3±4.4	21.3±3.3	27.7±5.8	26.3±4.7	22.7±2.4
glycine	7.1±1.5	7.7±5.9	7.7±3.1	5.7±1.1	5.9±1.7	5.0±1.1
L-glutamine	26.2±5.8	21.3±5.5	19.5±3.4	21.4±2.3	22.1±3.5	23.5±3.5
D-serine	0.8±0.3	0.2±0.1	0.8±0.1	2.7±1.1	0.6±0.1	0.4±0.2
L-serine	6.2±1.4	3.5±0.6	3.8±0.7	5.2±0.5	3.2±0.8	3.4±0.7

Changes in amine content. Very few changes were observed in amines in the regions studied both after drug withdrawal (Table 13, Figure 25A-C) and after abstinence from sucrose (Table 14, Figure 25D-F). The treatment x abstinence interaction caused some changes by region and substance in a dispersed manner. The only common change to both drugs of abuse, but not to sucrose, was the decrease in CeA of the L-glutamate/L-glutamine ratio after prolonged abstinence (Figure 25C). Specific changes to the abstinence of each of the substances were also observed. Cocaine consumption caused a decrease in L-glutamate in vmPFC that normalized after the month of abstinence (Figure 25A). Heroin abstinence occurred with an increase in the L-glutamate/D-serine ratio in dmPFC (Figure 25B). Sucrose consumption caused decreases in the L-glutamate/GABA ratio in BLA and shell taurine increases that normalized after the month of abstinence (Figures 25D, E), as well as decreases in L-aspartate levels in core after one month of abstinence (Figure 25F). A summary of the results is presented in Figure 26. Changes due to treatment effect are presented in Tables 15 (drugs) and 16 (sucrose).

Table 13. Level of amines (mean±SD) during the withdrawal of cocaine and heroin.

	1 day of withdrawal			30 days of withdrawal			ANOVA		
	saline	cocaine	heroin	saline	cocaine	heroin	$F(df_M,df_R)$	p	η^2
Central nucleus of amygdala:									
L-glutamate/L-glutamine	1.47±0.08	1.61±0.13*	1.56±0.32	1.53±0.14	1.38±0.07*f	1.43±0.10f	$F(2,38)=7.105$	.002	.272
Dorsomedial prefrontal cortex:									
L-glutamate/D-serine	75.36±18.74	85.20±30.20	62.13±8.77	72.30±12.15	66.96±10.86#	104.62±41.61#f	$F(2,34)=5.349$	.010	.239
Ventromedial prefrontal cortex:									
L-glutamate	46.94±4.17	42.14±2.96*	42.93±3.67	43.10±3.95f	47.53±3.51f	42.59±4.48	$F(2,40)=5.754$	.006	.223

Table 14. Level of amines (mean±SD) during the withdrawal of sucrose.

	1 day of withdrawal		30 days of withdrawal		ANOVA		
	water	sucrose	water	sucrose	$F(df_M,df_R)$	p	η^2
Basolateral amygdala:							
L-glutamate/GABA	1.90±0.27	1.72±0.17*	1.90±0.15	2.01±0.12f	$F(1,28)=4.763$	.038	.145
Nucleus accumbens core:							
L-aspartate	17.52±2.42	19.66±1.21	20.71±4.19f	17.58±1.86*	$F(1,29)=8.729$	.006	.231
D-serine	0.59±0.13	0.65±0.05	0.65±0.13	0.55±0.05	$F(1,28)=4.590$	.041	.141
L-glutamate/glycine	5.67±0.93	6.31±3.16	6.30±0.70	5.77±0.66	$F(1,29)=4.369$	.045	.131
Nucleus accumbens shell:							
taurine	22.65±2.39	28.85±3.07*	28.38±6.59f	25.49±1.96	$F(1,17)=7.145$	.016	.296

* p<.05 versus control
\# p<.05 between drugs
f p<.05 versus wd1 group

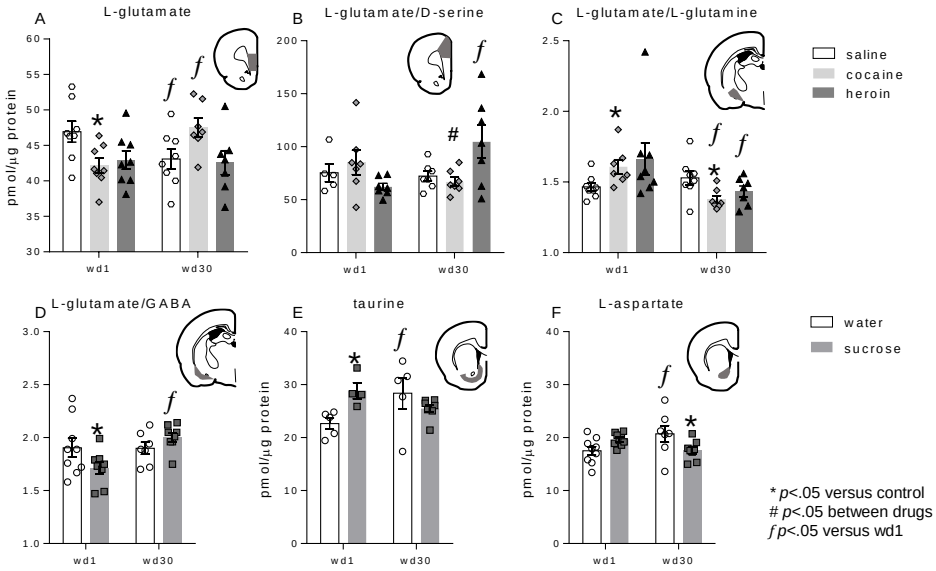


Figure 25. Changes in amines content during abstinence. Data of amine levels are presented for vmPFC (A), dmPFC (B) and CeA (C) during abstinence from cocaine and heroin use and for BLA (D), shell (E) and core (F) during abstinence from sucrose. The individual values are presented as well as the mean±SE.

Table 15. Perdurable effects of cocaine and heroin on brain amine content.

	1 day of withdrawal			30 days of withdrawal			ANOVA		
	saline	cocaine	heroin	saline	cocaine	heroin	$F(df_M,df_R)$	*p*	η^2
Nucleus accumbens core:									
L-glutamate	35.05±2.10	40.62±2.40*	35.99±1.31#	36.60±2.26	44.96±1.73*	39.38±2.05#	$F(2,37)=6.264$	.005	.253
L-aspartate	18.33±1.25	22.04±1.68*	20.05±0.91	18.04±0.93	23.69±1.95*	21.97±1.11	$F(2,38)=5.081$	.011	.211

Table 16. Perdurable effects of sucrose on brain amine content.

	1 day of withdrawal		30 days of withdrawal		ANOVA		
	water	sucrose	water	sucrose	$F(df_M,df_R)$	*p*	η^2
Central nucleus of amygdala:							
D-serine	0.22±0.05	0.41±0.10	0.20±0.04	0.34±0.06	$F(1,19)=4.701$	.043	.198
D-serine/L-serine	0.056±0.016	0.104±0.027	0.047±0.008	0.088±0.015	$F(1,18)=4.417$	.043	.208
L-glutamate/GABA	1.54±0.05	1.35±0.04	1.46±0.06	1.39±0.05	$F(1,30)=6.503$	.016	.178
L-glutamate/L-aspartate	1.95±0.05	1.76±0.05	1.84±0.05	1.78±0.06	$F(1,30)=5.419$	.027	.153
L-glutamate/D-serine	128.6±21.3	89.6±13.1	122.3±8.0	96.0±13.5	$F(1,17)=4.636$	.046	.214
Nucleus accumbens core:							
L-glutamate/GABA	1.79±0.06	2.06±0.07	1.90±0.09	2.03±0.07	$F(1,29)=7.313$	.011	.201

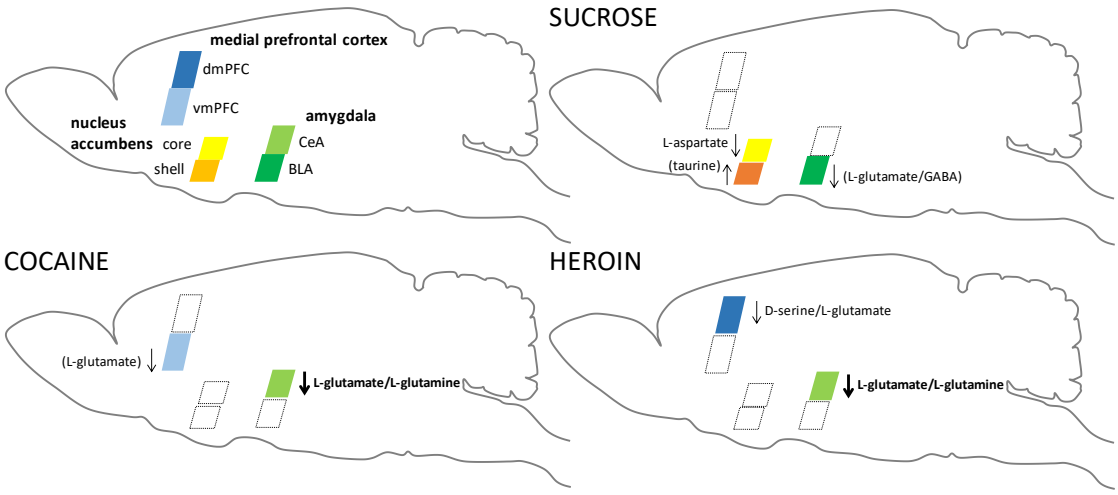


Figure 26. Summary of the results in levels of cerebral amines. Only those regions in which changes have been observed are colored. The changes between parentheses are those that occur in early abstinence and that revert after the month of abstinence.

3.4. GENE EXPRESSION OF GLUTAMATERGIC, GABAERGIC AND eCB SYSTEMS

Two different patterns of changes in gene expression were observed. Some genes increased their expression after a day of abstinence and then normalized one month later, and others suffered increases or decreases after a month of abstinence. Both changes are discussed below separately. The results are presented in Tables 17 (drugs) and 18 (sucrose). The perdurable effects of the treatment are presented in Tables 19 (drugs) and 20 (sucrose).

Gene expression changes in early withdrawal. Among the changes observed after heroin consumption we have an increase in the *Gabra1*/*Gabra2* ratio in BLA (Figure 27D) and *Actb* in core (Figure 27G). After self-administration of cocaine an increase in *Gria1* was observed in BLA (Figure 27A) and dmPFC (Figure 27E), accompanied by a slight increase in *Grin2a* and *Gria2* in BLA (Figure 27B, C) and in the ratio *Gria1*/*Gria2* in dmPFC (Figure 27F). The consumption of cocaine also caused an increase in the ratio *Gabrg2*/*Gabrd* in shell (Figure 27J) due to changes in the expression of these $GABA_A$ subunits (Figure 27H, I). Since the kinetic properties and location of the ionotropic glutamate and GABA receptors are determined by the composition of their subunits, these differences could be related to an altered synaptic activity.

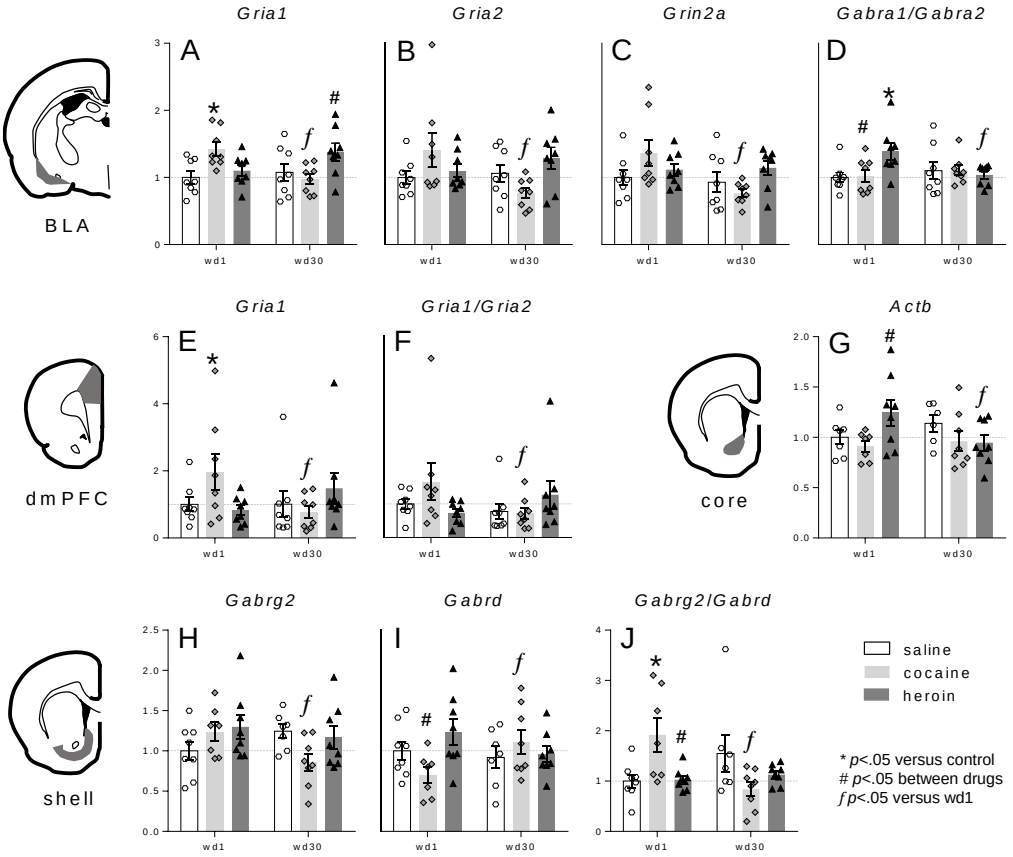


Figure 27. Gene expression after early abstinence. Changes in gene expression after the consumption of cocaine and heroin in BLA (A-D), dmPFC (E, F), core (G) and shell (H-J). The individual values are presented as well as the mean±SE.

Gene expression changes after protracted withdrawal. One-month abstinence after heroin consumption was associated with an increase in *Gabrd* in vmPFC (Figure 28E), and after cocaine consumption with an increase in the *Grin2a*/*Grin2b* ratio in dmPFC (Figure 28D). In addition, strong decreases in the expression of several genes in dmPFC were observed in the rats that consumed sucrose: *Actb*, *Grin1*, *Gria1*, *Gabra1*, *Gabra2* and the *Gabra1*/*Gabra2* ratio (Figure 28G-L). And it was after the month of abstinence that changes in endocannabinoid system genes were observed. On the one hand, increases of *Faah* and *Dagla* in BLA in rats that consumed heroin (Figure 28A, B), and *Napepld* decreases in shell in rats that consumed cocaine (Figure 28F). And both in rats that consumed heroin and in those that consumed cocaine, decreases in the *Napepld*/*Faah* ratio in BLA (Figure 28C). An increase in *Mgll* in CeA was also observed in the rats that consumed sucrose (Figure 28M). The different genes of the endocannabinoid system are responsible for the synthesis and degradation of anandamide (*Napepld* and *Faah*) and 2-arachidonylglycerol (*Dagla* and *Mgll*), both modulators of the glutamatergic and GABAergic systems. Therefore, these changes could mean changes in the modulating capacity of the excitatory and inhibitory synapses.

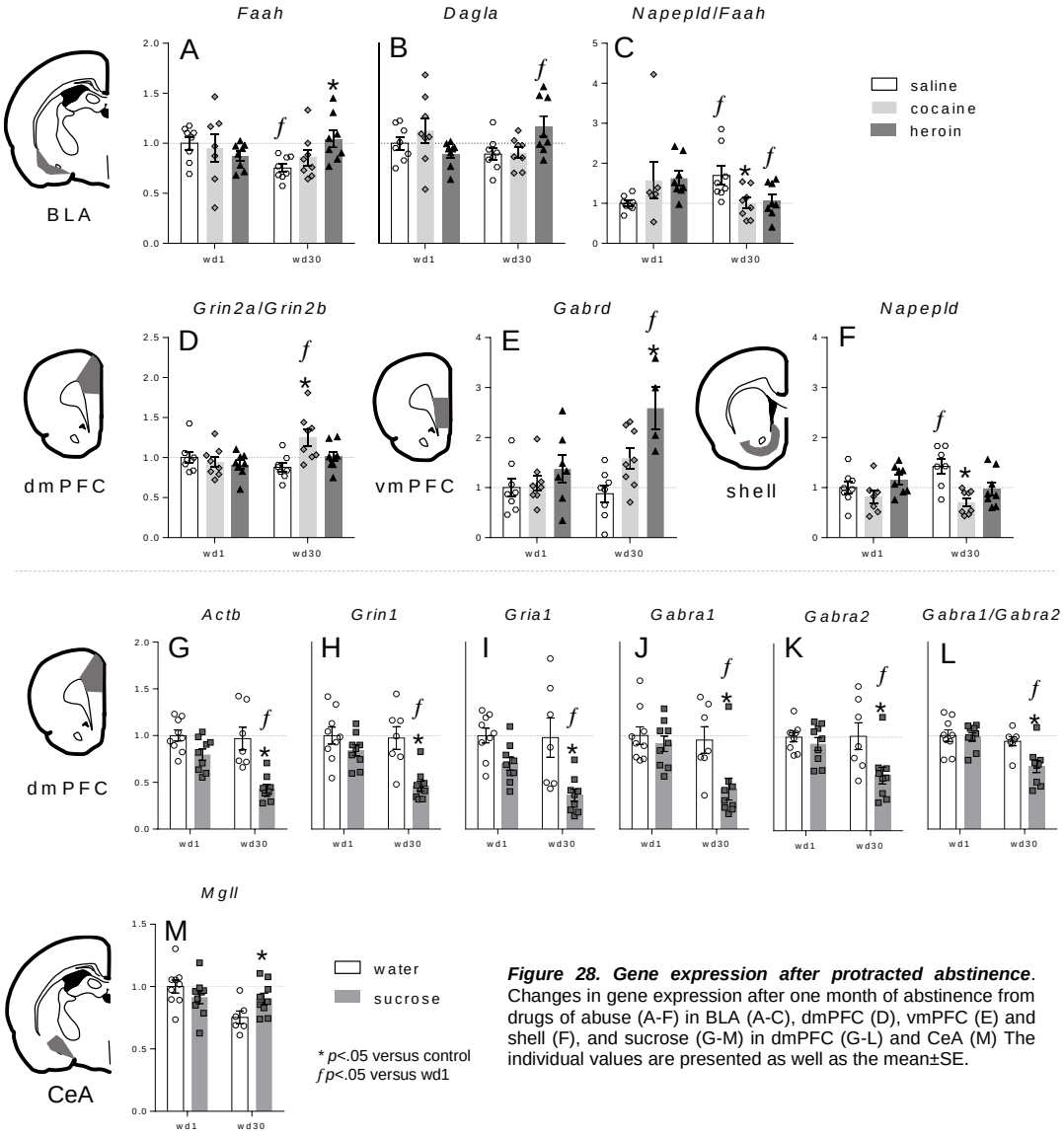


Figure 28. Gene expression after protracted abstinence. Changes in gene expression after one month of abstinence from drugs of abuse (A-F) in BLA (A-C), dmPFC (D), vmPFC (E) and shell (F), and sucrose (G-M) in dmPFC (G-L) and CeA (M) The individual values are presented as well as the mean±SE.

Table 17. Fold change (mean±SD) of gene expression during cocaine and heroin withdrawal.

	1 day of withdrawal			30 days of withdrawal			ANOVA		
	saline	cocaine	heroin	saline	cocaine	heroin	$F(df_M,df_R)$	p	η^2
Basolateral amygdala:									
Grin2a	1.00±0.32	1.37±0.55	1.12±0.26	0.93±0.42	0.77±0.16*f*	1.14±0.30	$F(2,42)=3.620$	.035	.131
Gria1	1.00±0.26	1.43±0.28*	1.10±0.23	1.08±0.37	0.98±0.21#*f*	1.38±0.37#a	$F(2,42)=6.461$	.004	.218
Gria2	1.00±0.27	1.40±0.72	1.10±0.29	1.06±0.37	0.77±0.22*f*	1.29±0.45	$F(2,42)=4.394$	.019	.165
Gabra1/Gabra2	1.00±0.21	1.02±0.26#	1.39±0.36*#*f*	1.10±0.37	1.11±0.21	1.04±0.15	$F(2,42)=3.507$	.039	.132
Napepld/Faah	1.00±0.19	1.58±1.20	1.63±0.50	1.70±0.66*f*	1.00±0.38*	1.08±0.43t*f*	$F(2,41)=6.587$	.003	.236
Dagla	1.00±0.17	1.13±0.35	0.90±0.13	0.89±0.17	0.91±0.15	1.17±0.28t*f*	$F(2,42)=4.952$	.012	.186
Faah	1.00±0.18	0.95±0.37	0.87±0.13	0.75±0.12*f*	0.86±0.22	1.04±0.23*	$F(2,41)=3.621$	.036	.144
Dorsomedial prefrontal cortex:									
Grin2a/Grin2b	1.00±0.19	0.95±0.19	0.91±0.15	0.88±0.15	1.25±0.30**f*	1.01±0.17	$F(2,42)=4.649$	.015	.158
Gria1	1.00±0.59	1.97±1.54	0.83±0.41	1.01±1.10	0.78±0.51*f*	1.48±1.33	$F(2,42)=3.310$	.046	.131
Gria1/Gria2	1.00±0.39	1.66±1.56	0.74±0.34	0.78±0.67	0.72±0.47*f*	1.28±1.18	$F(2,42)=3.148$	.053	.125
Ventromedial prefrontal cortex:									
Gabrd	1.00±0.50	1.09±0.42	1.38±0.72	0.88±0.47	1.59±0.59t#	2.59±0.85*#*f*	$F(2,37)=4.196$	0.023	.126
Nucleus accumbens core:									
Actb	1.00±0.19	0.81±0.32#	1.24±0.37#	1.14±0.20	0.98±0.30	0.94±0.22*f*	$F(2,38)=3.424$	.043	.138
Nucleus accumbens shell:									
Gabrd	1.00±0.33	0.70±0.27#	1.28±0.48#	0.92±0.36	1.11±0.43*f*	0.98±0.29	$F(2,38)=3.575$	.038	.150
Gabrg2	1.00±0.33	1.24±0.31	1.35±0.43	1.25±0.23	0.86±0.31*f*	1.21±0.41	$F(2,38)=3.195$	.052	.130
Gabrg2/Gabrd	1.00±0.36	1.92±0.90*#	1.04±0.23#	1.55±0.96	0.84±0.40*f*	1.17±0.18	$F(2,36)=6.903$	.003	.262
Napepld	1.00±0.33	2.52±4.85	1.19±0.29	1.42±0.40*f*	0.71±0.24*	1.00±0.39	$F(2,38)=3.727$	.033	.124

Table 18. Fold change (mean±SD) of gene expression during sucrose withdrawal.

	1 day of withdrawal		30 days of withdrawal		ANOVA		
	water	sucrose	water	sucrose	$F(df_M,df_R)$	p	η^2
Central nucleus of amygdala:							
Mgll	1.00±0.16	0.92±0.15	0.75±0.13*f*	0.90±0.13*	$F(1,29)=5.253$	.029	.133
Dorsomedial prefrontal cortex:							
Grin1	1.00±0.27	0.84±0.18	0.97±0.32	0.46±0.16**f*	$F(1,30)=4.717$	.038	.080
Gria1	1.00±0.24	0.70±0.21	0.98±0.55	0.36±0.19**f*	$F(1,30)=4.128$	.051	.064
Actb	1.00±0.18	0.80±0.18	0.97±0.32	0.43±0.13**f*	$F(1,30)=10.221$	.003	.111
Gabra1	1.00±0.28	0.91±0.25	0.95±0.38	0.43±0.35**f*	$F(1,30)=4.054$	.053	.083
Gabra2	1.00±0.15	0.93±0.21	1.01±0.38	0.59±0.27**f*	$F(1,30)=4.622$	.040	.091
Gabra1/Gabra2	1.00±0.18	0.99±0.13	0.94±0.13	0.67±0.20**f*	$F(1,30)=4.861$	.035	.092

* p<.05 versus control
t p<.10 versus control
\# p<.05 between drugs
f p<.05 versus wd1
a p<.10 versus wd1

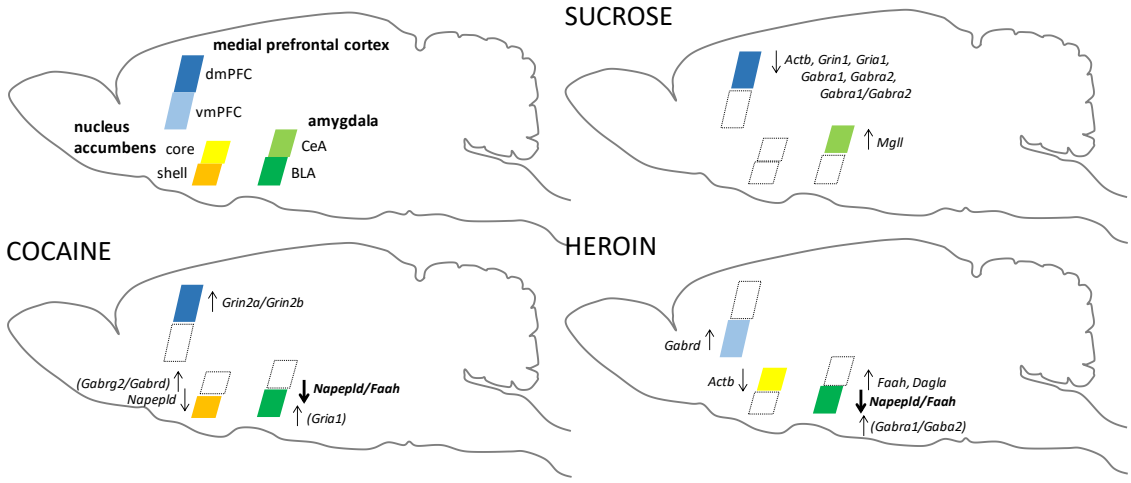


Figure 29. Summary of the results in gene expression. Only those regions in which changes have been observed are colored. The changes between parentheses are those that occur in early abstinence and that revert after the month of abstinence.

Table 19. Perdurable changes (mean±SD) of gene expression after cocaine and heroin consumption.

	1 day of withdrawal			30 days of withdrawal			ANOVA		
	saline	cocaine	heroin	saline	cocaine	heroin	$F(df_M,df_R)$	*p*	η^2
Dorsomedial prefrontal cortex:									
Cnr1	1.00±0.18	0.88±0.09*	0.89±0.10t	1.05±0.23	0.90±0.12*	0.90±0.11t	*F*(2,42)=4.200	.022	.165
Ventromedial prefrontal cortex:									
Grin1	1.00±0.21	1.05±0.22	0.84±0.27*	1.11±0.32	1.00±0.25	0.80±0.23*	*F*(2,42)=4.064	.024	.159
Gabra1	1.00±0.33	0.86±0.22#	0.71±0.11*#	1.06±0.30	0.99±0.20#	0.65±0.30*#	*F*(2,42)=7.890	.001	.267
Gabrd	1.00±0.50	1.09±0.42*#	1.38±0.72*#	0.88±0.47	1.59±0.59*#	2.59±0.85*#	*F*(2,37)=10.179	.0003	.306
Gabra1/Gabra2	1.00±0.31	1.03±0.27*#	0.75±0.16*#	0.98±0.18	0.94±0.35*#	0.63±0.19*#	*F*(2,42)=7.185	.002	.248
Gabrg2/Gabrd	1.00±0.86	0.84±0.44	0.70±0.51*	2.42±3.47	0.78±0.60	0.33±0.14*	*F*(2,37)=4.813	.014	.195
Actb	1.00±0.23	0.98±0.15#	0.87±0.10*#	1.05±0.10	1.12±0.12#	0.83±0.14*#	*F*(2,42)=8.535	.001	.269
Faah	1.00±0.21	0.99±0.30	1.38±0.52*	0.91±0.22	1.19±0.24	1.39±0.47*	*F*(2,42)=6.194	.004	.221
Nucleus accumbens shell:									
Grin2a	1.00±0.36	1.64±1.25	1.72±0.80*	0.87±0.20	1.03±0.44	1.28±0.52*	*F*(2,38)=4.777	.014	.187
Grin2b	1.00±0.40	1.20±0.64#	1.69±0.68*#	0.95±0.35	1.04±0.23#	1.34±0.47*#	*F*(2,39)=4.841	.013	.190
Gria1	1.00±0.22	1.24±0.50	1.63±0.64*	0.85±0.18	1.02±0.39	1.30±0.59*	*F*(2,38)=5.266	.010	.204
Gria1/Gria2	1.00±0.31	1.09±0.46t	1.11±0.35	0.69±0.17	1.45±0.79t	1.15±0.44	*F*(2,38)=3.223	.051	.133
Actb	1.00±0.22	1.12±0.22#	1.40±0.42*#	1.07±0.22	0.92±0.22#	1.22±0.19*#	*F*(2,39)=5.918	.006	.213
Napepld	1.00±0.33	2.52±4.85*#	1.19±0.29#	1.42±0.40	0.71±0.24*#	1.00±0.39#	*F*(2,38)=7.374	.002	.246
Faah	1.00±0.12	1.18±0.24*	1.23±0.06	1.03±0.23	1.33±0.24*	1.08±0.16	*F*(2,38)=5.722	.007	.212
Napepld/Faah	1.00±0.42	0.68±0.27*#	0.94±0.20#	1.35±0.31	0.53±0.19*#	0.91±0.34#	*F*(2,38)=13.487	.00004	.382

Table 20. Perdurable changes (mean±SD) of gene expression after sucrose consumption.

	1 day of withdrawal		30 days of withdrawal		ANOVA		
	water	sucrose	water	sucrose	$F(df_M,df_R)$	*p*	η^2
Dorsomedial prefrontal cortex:							
Grin1	1.00±0.27	0.84±0.18	0.97±0.32	0.46±0.16	*F*(1,30)=17.100	.0003	.290
Grin2a	1.00±0.26	0.63±0.17	0.95±0.60	0.33±0.25	*F*(1,30)=19.920	.0001	.317
Grin2b	1.00±0.23	0.54±0.16	0.85±0.54	0.25±0.16	*F*(1,27)=13.009	.001	.286
Gria1	1.00±0.24	0.70±0.21	0.98±0.55	0.36±0.19	*F*(1,30)=19.732	.0001	.308
Gria1/Gria2	1.00±0.18	0.70±0.20	0.94±0.41	0.39±0.18	*F*(1,30)=24.329	.00003	.391
Gabra1	1.00±0.28	0.91±0.25	0.95±0.38	0.43±0.35	*F*(1,30)=7.924	.009	.162
Gabra2	1.00±0.15	0.93±0.21	1.01±0.38	0.59±0.27	*F*(1,30)=8.695	.006	.171
Gabra1/Gabra2	1.00±0.18	0.99±0.13	0.94±0.13	0.67±0.20	*F*(1,30)=5.889	.021	.112
Actb	1.00±0.18	0.80±0.18	0.97±0.32	0.43±0.13	*F*(1,30)=34.344	.00000	.372
Dagla	1.00±0.14	0.93±0.19	1.05±0.26	0.80±0.18	*F*(1,30)=5.917	.021	.155
Mgll	1.00±0.13	0.78±0.15	0.86±0.16	0.57±0.10	*F*(1,30)=30.444	.00001	.389
Dagla/Mgll	1.00±0.14	1.19±0.16	1.22±0.24	1.39±0.25	*F*(1,30)=6.613	.015	.141
Nucleus accumbens core:							
Gria2	1.00±0.22	0.83±0.19	1.12±0.18	0.97±0.16	*F*(1,30)=5.636	.024	.144
Napepld	1.00±0.16	0.79±0.37	1.15±0.23	0.84±0.22	*F*(1,30)=8.765	.006	.220
Nucleus accumnes shell:							
Grin1	1.00±0.16	0.95±0.26	1.12±0.10	0.83±0.25	*F*(1,22)=4.103	.055	.150
Grin2a/Grin2b	1.00±1.00	0.51±0.61	0.50±0.59	0.11±0.07	*F*(1,22)=7.600	.012	.215
Gabrg2	1.00±0.15	0.77±0.25	1.00±0.29	0.71±0.15	*F*(1,22)=9.267	.006	.294
Gabrd	1.00±0.24	1.84±0.58	1.09±0.26	1.65±0.40	*F*(1,22)=17.801	.0004	.422
Gabrg2/Gabrd	1.00±0.31	0.43±0.18	0.94±0.47	0.41±0.08	*F*(1,22)=28.741	.00002	.547
Cnr1	1.00±0.17	1.57±0.43	0.93±0.19	1.27±0.40	*F*(1,22)=12.030	.002	.315
Faah	1.00±0.41	1.58±0.57	1.02±0.37	1.70±0.55	*F*(1,22)=10.347	.004	.318

* *p*<.05 versus control
\# *p*<.05 between drugs

Gene expression covariation by PCA. When performing principal component analysis (PCA) we found two latent variables affected by abstinence: F1, downregulated after cocaine seeking incubation ($F(2,41)=3.313$, $p=.046$, $\eta^2=.128$; effect of abstinence in animals that consumed cocaine: $p=.007$), and F2, upregulated after heroin seeking incubation ($F(2,41)=5.177$, $p=.010$, $\eta^2=.198$; effect of the abstinence in animals that consumed heroin: $p=.021$). Other latent variables, affected by the different treatments, are presented in Tables 21 (drugs) and 22 (sucrose). The composition of all the latent variables (or major components) extracted from each structure are described in Figures 30 (drugs) and 31 (sucrose).

Table 21. Perdurable changes in principal components after cocaine and heroin consumption.

	saline wd1		cocaine wd1		heroin wd1		saline wd30		cocaine wd30		heroin wd30	
	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*
F6[a]	0.067	*1.238*	-0.067	*0.580*	-0.563	*0.584*	0.590	*1.136*	0.629	*0.901*	-0.655	*0.830*
F11[b]	-0.414	*0.611*	0.265	*0.906*	0.996	*1.459*	-0.793	*0.653*	-0.260	*0.490*	0.302	*0.823*
F12[c]	-0.044	*0.837*	-0.354	*0.972*	0.460	*0.669*	0.999	*0.854*	-0.894	*0.734*	-0.034	*0.987*

[a]ANOVA: $F(2,47)=5.356$, $p=.008$, $\eta^2=.190$; *post hoc*: saline *vs* heroin, $p=.017$, cocaine *vs* heroin $p=.026$.

[b]ANOVA: $F(2,43)=7.521$, $p=.002$, $\eta^2=.263$; *post hoc*: saline *vs* heroin, $p=.001$.

[c]ANOVA: $F(2,43)=6.854$, $p=.003$, $\eta^2=.229$; *post hoc*: saline *vs* cocaine, $p=.003$, cocaine *vs* heroin $p=.035$.

Table 22. Perdurable changes in principal components after sucrose consumption.

	water wd1		water wd30		sucrose wd1		sucrose wd30	
	mean	*SD*	mean	*SD*	mean	*SD*	mean	*SD*
F'7[a]	0.955	*0.593*	0.473	*0.998*	-0.188	*0.375*	-1.134	*0.42*
F'13[b]	0.14	*0.544*	0.76	*0.944*	-0.659	*1.229*	-0.072	*0.793*
F'14[c]	-0.775	*0.411*	-0.814	*0.752*	0.826	*0.783*	0.481	*0.783*

[a]ANOVA: $F(1,33)=42.104$, $p=.000$, $\eta^2=.481$.

[b]ANOVA: $F(1,33)=6.762$, $p=.014$, $\eta^2=.169$.

[c]ANOVA: $F(1,25)=27.335$, $p=.000$, $\eta^2=.529$.

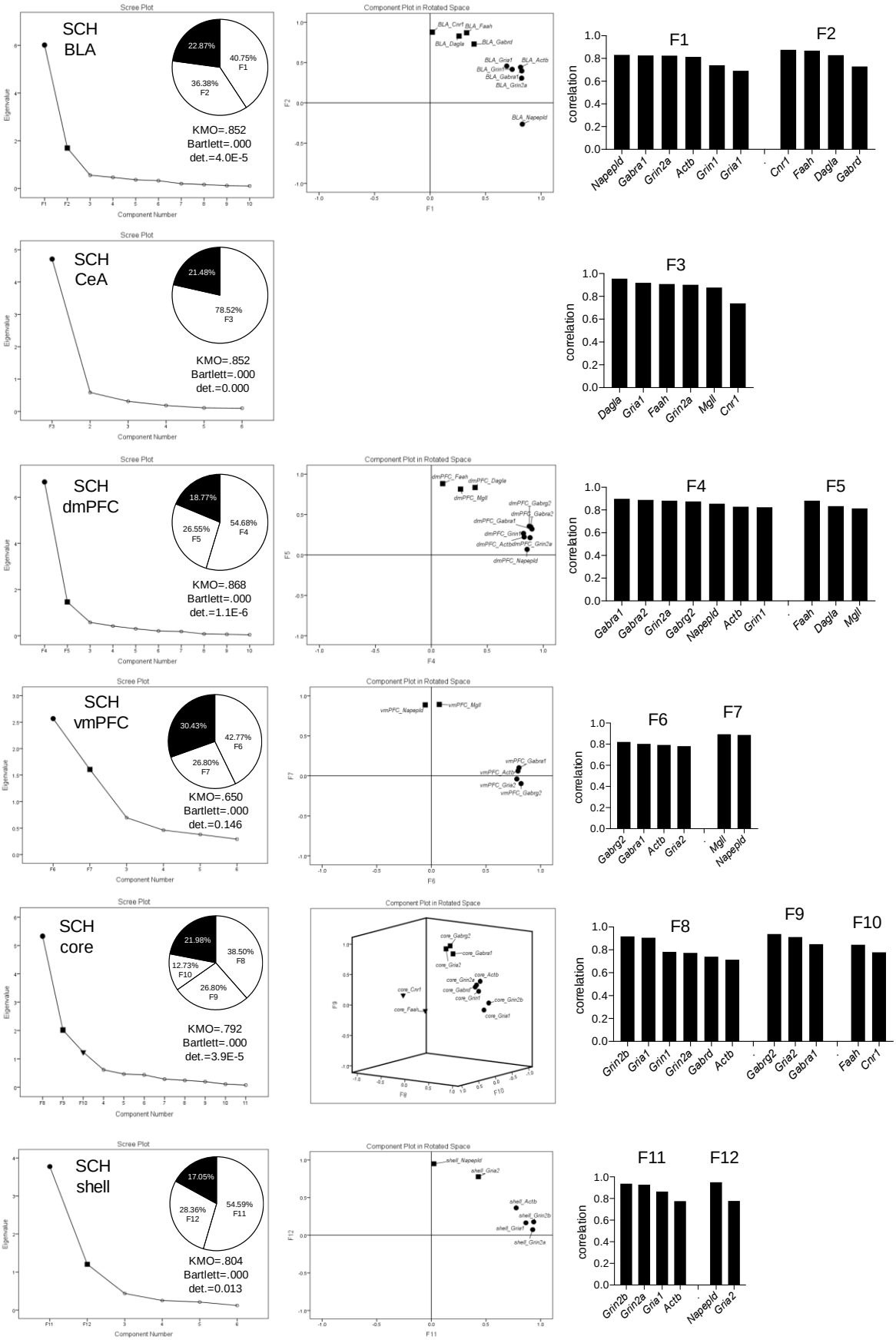


Figure 30. Characteristics of the components in SCH experiment.

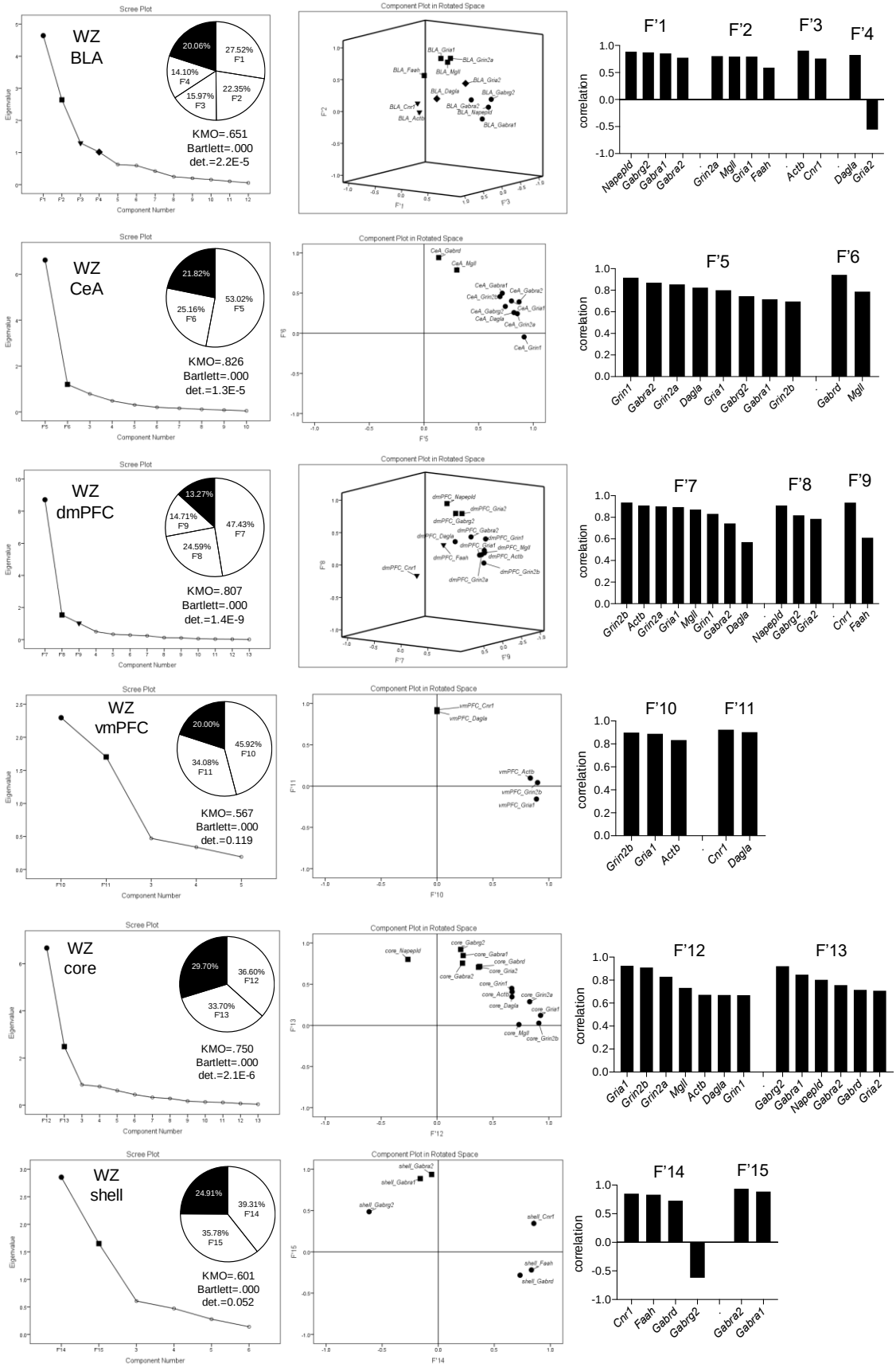
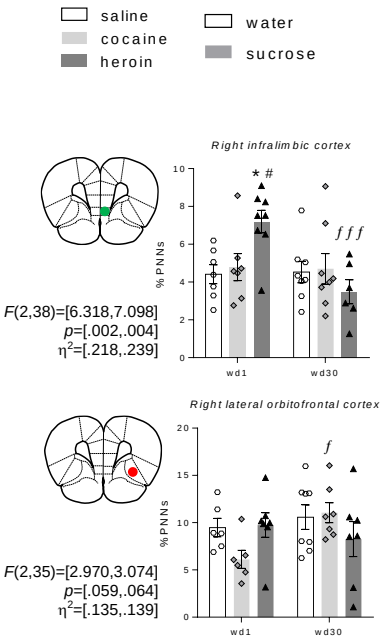


Figure 31. Characteristics of the components in WZ experiment.

3.5. PERINEURONAL NETS IN THE PREFRONTAL CORTEX

Levels of perineuronal nets (PNN) by cortical region. An effect of early abstinence after heroin use (significant) and after cocaine use (trend) that reversed after the month of abstinence was observed. Heroin consumption caused an increase in PNNs concentrated in the right infralimbic cortex (greater than the control group, p=[.007, .014], and that the cocaine group, p=[.023, .032]) that reversed after one month (greater than in one-month heroin abstinence rats, p<.001) (Figure 32, *top*). Cocaine consumption caused a decrease in PNNs concentrated in the right lateral orbitofrontal cortex, which reverted after the month of abstinence (p=[.014, .015] with respect to cocaine one-month abstinence rats (Figure 32, *bottom*).



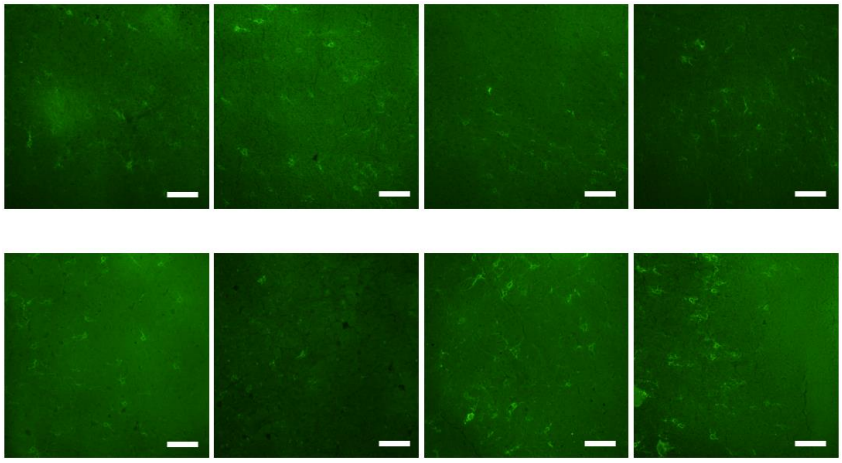
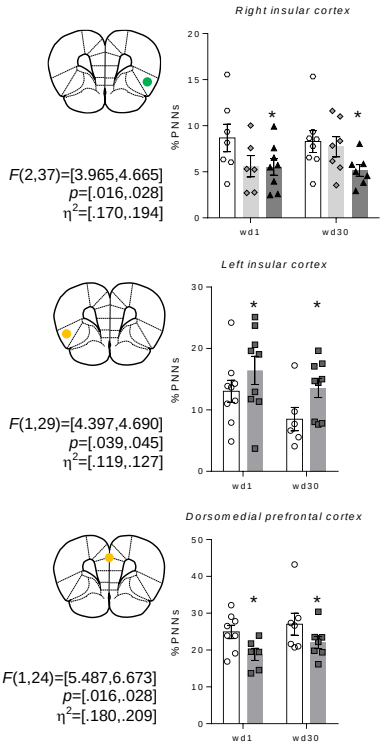


Figure 32. Changes in perineuronal net density during withdrawal.

* p<.05 versus control
\# p<.05 between drugs
ƒ p<.05 versus wd1
ƒƒƒ p<.001 versus wd1

Perdurable effects of heroin and sucrose consumption were also observed. While heroin consumption caused a decrease in the number of PNNs in the right insular cortex (Figure 33, *top*), the consumption of sucrose caused an increase in the left insular cortex (Figure 33, *middle*) in detriment of the dorsomedial region, comprised by the left and right regions of ACC and dPL (Figure 33, *bottom*).



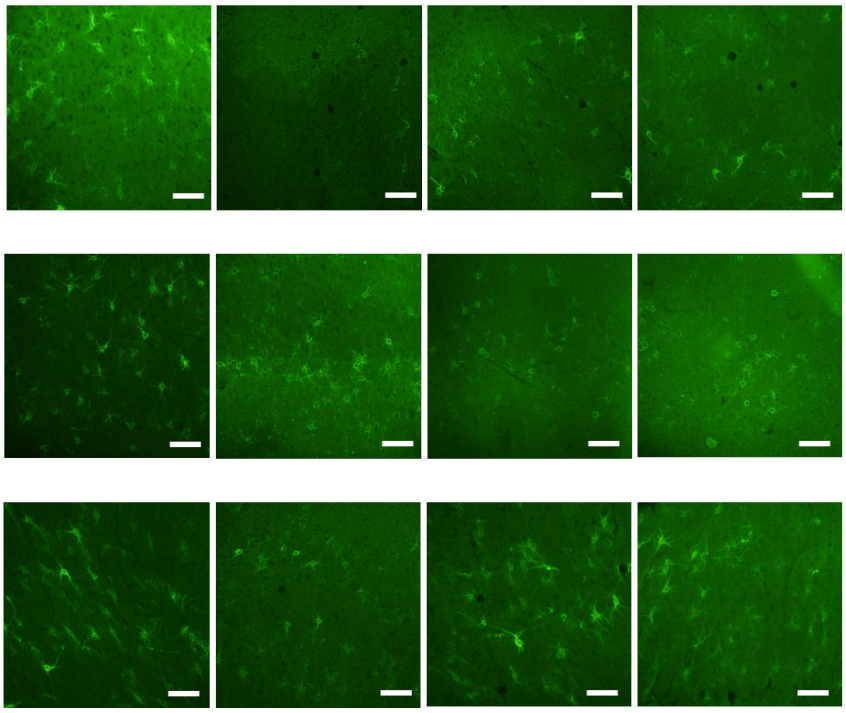
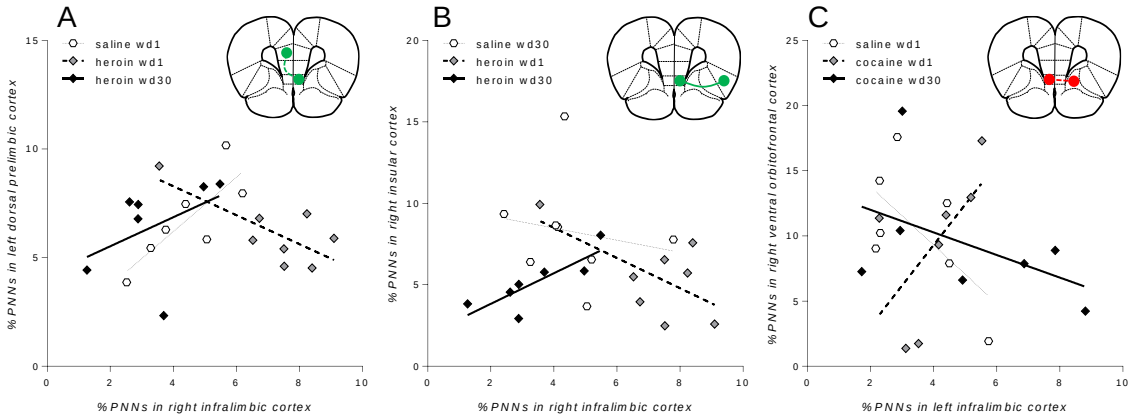


Figure 33. Perdurable changes in perineuronal net density.

Correlations of PNN levels between cortical regions. Next, we studied if the number of PNNs correlated between regions and, more importantly, if these correlations changed throughout abstinence. Clear effects were found in the rats that consumed heroin, specifically in the prefrontal cortical areas that correlated with the number of PNNs in the right IL cortex. After a day of abstinence, the rats showed a negative correlation between this area and left dPL, not present in the saline controls or in the rats after one month of abstinence (Figure 34A), which nevertheless presented a negative correlation between the PNNs of right IL and right insular cortices, not present in the control rats or after only one day of abstinence (Figure 34B). A weaker effect was found in the rats that consumed cocaine after a day of abstinence, this time between the left IL cortex and the right vOFC region (Figure 34C). More specifically, the positive correlation was absent in control rats after one month of abstinence. In addition, less clear effects were found in rats that consumed heroin (Figure 34D) and in those that consumed sucrose (Figure 34E, F). Such effects could be due to artifacts due to the presence of possible extreme values. Figure 34 shows the values of the Pearson (r) or Spearman (ρ) correlations, as well as the contrasts between experimental groups ($Z_{\text{difference}}$).



$r_{saline\ wd1}$=+.813, p=.026
$r_{heroin\ wd1}$=-.742, p=.035
$r_{heroin\ wd30}$=+.429, p=.336

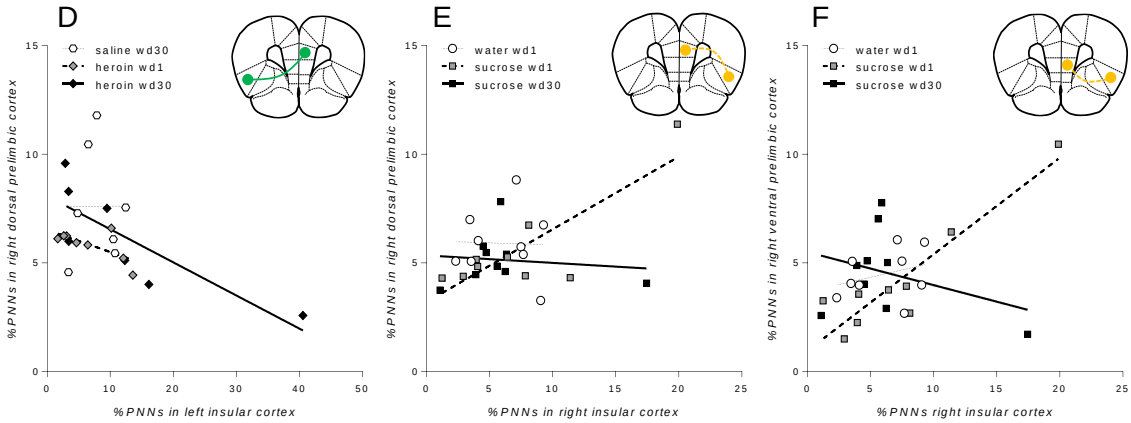
$Z_{wd1,\ heroin\ vs\ saline}$=3.116, p=.0018
$Z_{heroin,\ wd1\ vs\ wd30}$=2.109, p=.035

$\rho_{saline\ wd30}$=-.333, p=.420
$\rho_{heroin\ wd1}$=-.310, p=.456
$\rho_{heroin\ wd30}$=+.893, p=.0068

$Z_{wd30,\ heroin\ vs\ saline}$=2.657, p=.0079
$Z_{heroin,\ wd30\ vs\ wd1}$=2.617, p=.0089

$\rho_{saline\ wd1}$=-.464, p=.294
$\rho_{cocaine\ wd1}$=+.786, p=.036
$\rho_{cocaine\ wd30}$=-.393, p=.383

$Z_{wd1,\ cocaine\ vs\ saline}$=2.210, p=.027
$Z_{cocaine,\ wd1\ vs\ wd30}$=2.086, p=.037



$\rho_{saline\ wd30}$=-.500, p=.207
$\rho_{heroin\ wd1}$=-.563, p=.146
$\rho_{heroin\ wd30}$=-.964, p=.0005

$Z_{wd30,\ heroin\ vs\ saline}$=2.168, p=.030
$Z_{heroin,\ wd30\ vs\ wd1}$=2.037, p=.042

$r_{water\ wd1}$=-.032, p=.934
$r_{sucrose\ wd1}$=+.827, p=.006
$r_{sucrose\ wd30}$=-.129, p=.741

$Z_{wd1,\ sucrose\ vs\ water}$=2.096, p=.036
$Z_{sucrose,\ wd1\ vs\ wd30}$=2.265, p=.024

$r_{water\ wd1}$=+.311, p=.415
$r_{sucrose\ wd1}$=+.922, p=.0004
$r_{sucrose\ wd30}$=-.345, p=.364

$Z_{wd1,\ sucrose\ vs\ water}$=2.220, p=.026
$Z_{sucrose,\ wd1\ vs\ wd30}$=3.400, p=.0007

Figure 34. Changes in the correlation between density of perineuronal nets of different regions. The statistical parameters of the correlations are indicated, as well as the location of the regions involved and whether they are changes after early (dashed lines) or late (continuous lines) abstinence.

3rd Goal: CHANGES IN STRESS RELATED PARAMETERS DURING SEEKING INCUBATION

3.6. PERIPHERAL PARAMETERS

Weight of animal, liver and spleen. An expected weight gain was observed in the animals during the month of withdrawal (Figure 35A). The splenic index was higher in the intravenous administration groups than in the oral administration groups, possibly due to the effect of catheterization. In addition, an increase in the spleen after cocaine consumption was observed ($F(2,40)=4.973$, $p=.012$, $\eta^2=.071$, Figure 35C, *left*).

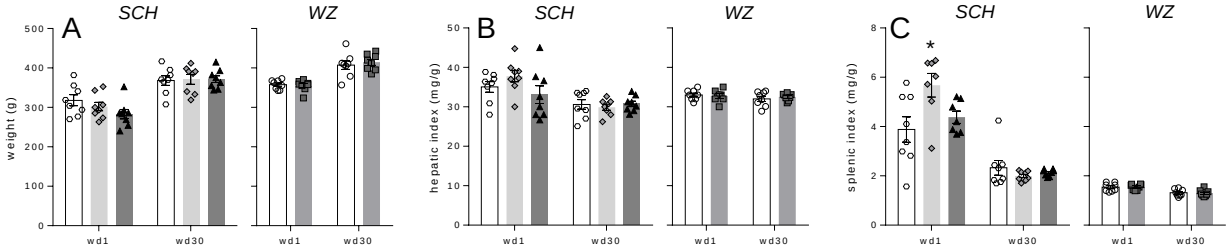


Figure 35. Weight evolution. The weights of the rats (A), their hepatic indices (B) and splenic indices (C) are presented. Mean±SE.

Adrenal glands and plasmatic corticosterone. Adrenal hyperplasia was observed after self-administration sessions of drugs of abuse ($F(2,41)=6.570$, $p=.003$, $\eta^2=.073$, Figure 36A, *left*), accompanied by elevation, in some animals, of corticosterone levels (time effect only: $F(1,39)=5.239$, $p=.028$, $\eta^2=.106$, Figure 36B, *left*). An increase in plasma corticosterone levels was observed between the wd1 and wd30 groups of sucrose ($F(1,29)=5.822$, $p=.022$, $\eta^2=.147$, Figure 36B, *right*). The average value of corticosterone in the saline and water groups was 50.4±46.9 and 30.4±32.2 ng/mL plasma (mean±SD).

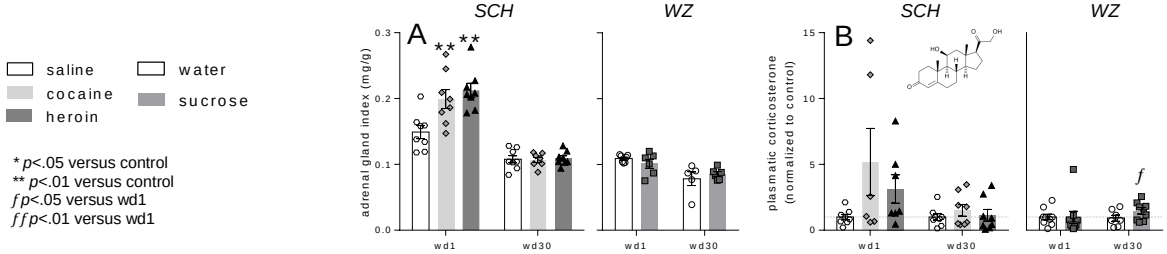


Figure 36. Adrenal glands and corticosterone. The adrenal glandular indexes (A) and the relative levels of plasma corticosterone (B) are presented. Mean±SE.

Levels of plasmatic amines. The consumption of both cocaine and heroin caused a decrease in the plasma L-glutamine/L-ornithine ratio, and abstinence occurred with an increase in its value in both groups ($F(2,38)=6.107$, $p=.005$, $\eta^2=.197$; Figure 37). The consumption of sucrose did not cause any change in the plasma levels of the studied amines. Table 23 shows the absolute data (pmol/mL of plasma) of each amine in both experiments.

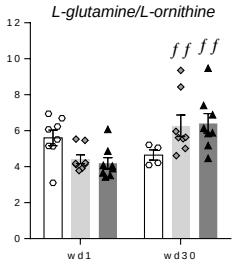


Figure 37. Ratio L-glutamine/L-ornithine.

Table 23. Levels of plasma amines in both experiments (mean±SD, N).

	SCH experiment		WZ experiment	
glycine	404,8 ± 236,8	47	490,6 ± 77,8	36
L-alanine	531,5 ± 146,8	47	792,3 ± 146,9	37
L-glutamate	108,3 ± 26,1	47	108,5 ± 17,9	29
L-glutamine	689,0 ± 145,3	47	976,0 ± 133,4	34
L-isoleucine	110,3 ± 26,8	47	234,3 ± 42,4	34
L-ornithine	131,4 ± 24,5	47	119,8 ± 17,2	37
L-proline	293,5 ± 47,0	47	441,1 ± 95,8	37
L-serine	365,6 ± 59,1	47	632,4 ± 92,1	37
L-threonine	517,6 ± 81,6	47	689,3 ± 104,7	37
taurine	409,8 ± 97,6	47	617,6 ± 141,6	37

3.7. NORADRENERGIC RECEPTORS

Perdurable gene expression changes. We observe lasting changes both after heroin and cocaine (Table 24) and after of sucrose consumption (Table 25).

		1 day of withdrawal			30 days of withdrawal			ANOVA		
		saline	cocaine	heroin	saline	cocaine	heroin	$F(df_M,df_R)$	p	η^2
Adra2a/Adrb1	dmPFC	1.08±0.36	1.50±0.65	1.57±0.49*	1.32±0.51	1.25±0.35	2.13±0.78*	$F(2,42)=5.948$	.005	.199
Adra1/Adrb1	dmPFC	0.97±0.38	1.06±0.62	1.41±0.58t	1.13±0.47	1.08±0.63	1.50±0.37t	$F(2,42)=3.153$	.053	.129
Adra2a	shell	1.00±0.42	0.93±0.27*	1.17±0.27	1.18±0.22	0.75±0.21*	0.98±0.25	$F(2,38)=3.528$	.039	.142

Table 24. *Adr* expression after drugs consumption.

		1 day of withdrawal		30 days of withdrawal		ANOVA		
		water	sucrose	water	sucrose	$F(df_M,df_R)$	p	η^2
Adra1	dmPFC	1.00±0.26	0.66±0.29	0.75±0.25	0.53±0.23	$F(1,30)=9.880$	.004	.218
	core	1.00±0.40	0.60±0.24	1.14±0.57	0.75±0.23	$F(1,30)=8.402$	.007	.213
Adra2a	dmPFC	1.00±0.22	0.82±0.18	0.88±0.35	0.50±0.37	$F(1,30)=8.465$	.007	.175
	BLA/dmPFC	1.03±0.29	1.47±0.43	2.35±0.74	5.76±4.76	$F(1,22)=8.766$	.007	.141
Adrb1	dmPFC	1.00±0.14	0.86±0.16	0.88±0.19	0.43±0.23	$F(1,30)=21.458$	.00007	.270
	dmPFC/vmPFC	1.02±0.23	0.91±0.20	1.07±0.51	0.48±0.36	$F(1,30)=9.294$	.005	.198
	BLA/dmPFC	0.96±0.24	1.25±0.37	1.38±0.30	4.22±2.54	$F(1,22)=20.416$	.0002	.267
Adra1/Adrb1	core	1.03±0.37	0.79±0.53	1.16±0.51	0.66±0.22	$F(1,30)=7.151$	.012	.191

Table 25. *Adr* expression after sucrose consumption.

Gene expression changes during the abstinence. Only after heroin and sucrose consumption were changes in the expression of adrenergic receptors found. After one month of heroin withdrawal rats exhibit an increase in the expression of *Adrb1* in BLA ($F(2,41)=3.538$, $p=.038$, $\eta^2=.141$, Figure 38A). In the rats that consumed sucrose we observe a decrease in the expression of *Adrb1* in dmPFC ($F(1,30)=5.980$, $p=.021$, $\eta^2=.075$, Figure 38B). In addition, the relative expression of *Adrb1* in dmPFC with respect to its expression in vmPFC and BLA also fell (dmPFC/vmPFC: $F(1,30)=4.309$, $p=.047$, $\eta^2=.092$; BLA/dmPFC: $F(1,22)=7.264$, $p=.013$, $\eta^2=.095$, Figure 38C, D).

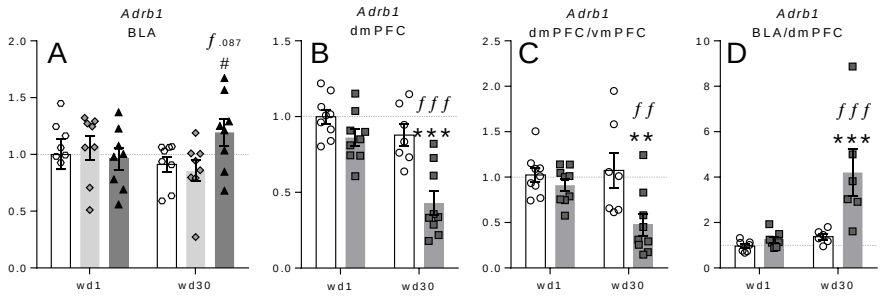
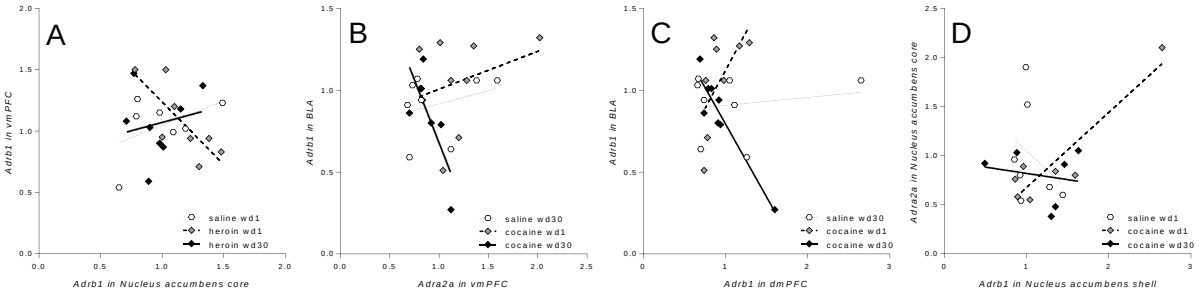


Figure 38. Changes in Adr expression during withdrawal. Changes in heroin (A) and sucrose groups (B-D). Mean±SE.

** $p<.01$ versus control
*** $p<.001$ versus control
# $p<.05$ between drugs
$f_{.087}$ $p=.087$ versus wd1
ff $p<.01$ versus wd1
fff $p<.001$ versus wd1

Correlations between brain regions. Next we compared correlations of these expressions between groups. After early heroin abstinence, a negative correlation was found between the expression of *Adrb1* in vmPFC and core, not present in the control group or after one month of abstinence (Figure 39A). After a month of abstinence from cocaine, a negative correlation was observed between the expression of *Adra2a* in vmPFC and *Adrb1* in BLA, neither present in the control group nor after a day of abstinence (Figure 39B). Two other effects are also presented, possibly due to extreme values (Figure 39C, D).



$r_{saline\ wd1}=+.463$, $p=.295$
$r_{heroin\ wd1}=-.788$, $p=.020$
$r_{heroin\ wd30}=+.206$, $p=.625$

$r_{saline\ wd30}=+.302$, $p=.468$
$r_{cocaine\ wd1}=+.286$, $p=.492$
$r_{cocaine\ wd30}=-.760$, $p=.029$

$r_{saline\ wd30}=+.177$, $p=.676$
$r_{cocaine\ wd1}=+.624$, $p=.098$
$r_{cocaine\ wd30}=-.940$, $p=.0005$

$r_{saline\ wd1}=-.324$, $p=.478$
$r_{cocaine\ wd1}=+.918$, $p=.004$
$r_{cocaine\ wd30}=-.181$, $p=.732$

$Z_{wd1,\ heroin\ vs\ saline}=2.337$, $p=.019$
$Z_{heroin,\ wd1\ vs\ wd30}=2.016$, $p=.044$

$Z_{wd30,\ cocaine\ vs\ saline}=2.068$, $p=.039$
$Z_{cocaine,\ wd30\ vs\ wd1}=2.040$, $p=.041$

$Z_{wd30,\ cocaine\ vs\ saline}=3.03$, $p=.002$
$Z_{cocaine,\ wd30\ vs\ wd1}=3.91$, $p=.0001$

$Z_{wd1,\ cocaine\ vs\ saline}=2.704$, $p=.007$
$Z_{cocaine,\ wd1\ vs\ wd30}=2.303$, $p=.021$

Figure 39. Changes in the correlation between gene expression of adrenergic receptors. Below the graph are the statistical parameters of the correlations after heroin (A) and cocaine (B-D) intake.

4. DISCUSION

Picture by Roberto Maján

1st Goal: SELF-ADMINISTRATION AND SEEKING INCUBATION

4.1. BEHAVIOURAL PREDICTIVE VARIABLES OF SEEKING INCUBATION

One of the aims of the work was to find predictive variables of the incubation during the self-administration sessions and then detect biochemical variables that correlate with them. To do this, we performed correlations between behavioural parameters of the self-administration sessions and parameters of the extinction tests in those rats that underwent both tests (wd1 and wd30). We did not find any variable or any combination of variables (using PCA) that correlated enough with the calculated incubation rates. With a different paradigm, also with a larger sample size, Kasanetz *et al*. (2010) classified rat as addict or non-addict according to a criterion adapted from the DSM (implemented by Belin *et al*., 2009), by subjecting the rats to certain diagnostic tests and searching, during 15min unreinforced sessions intercalated throughout the self-administration sessions, differential behaviour patterns between both groups with those who calculate an addiction score. With this score, they then classify new rats as resistant or vulnerable to addiction, without having to submit them to diagnostic tests (Figure 40). It is possible, therefore, that in our case the sample size is not high enough, so this objective can be pursued by increasing the sample size with future experiments of seeking incubation.

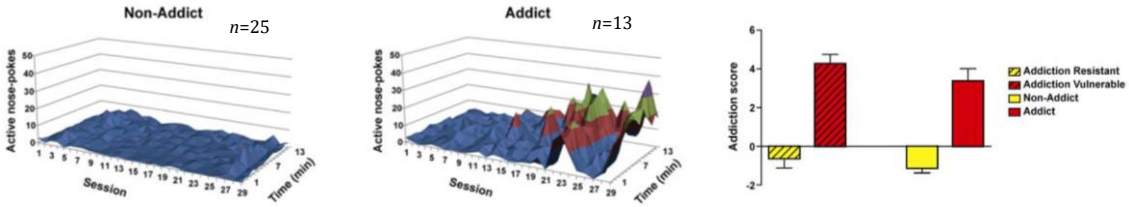


Figure 40. Differential behaviour patterns during non-reinforced sessions. Based on the behaviour during these sessions, they calculate an addiction score by which they are able to classify the rats as resistant or vulnerable to addiction. (Kasanetz *et al*., 2010).

2nd Goal: CHANGES IN GLUTAMATERGIC AND GABAERGIC PARAMETERS DURING SEEKING INCUBATION

Since our may hypothesis was that the phenomenon of incubation of seeking has a common substrate regardless of the substance to which the individual is abstemious, we wanted to trace parallel equivalent changes in abstaining rats of the three substances studied (cocaine, heroin and sucrose) in parameters that gave us information about the state of activity and about plastic phenomena in the studied regions (nucleus accumbens core and shell, basolateral amygdala, central nucleus of the amygdala, dorsomedial and ventromedial prefrontal cortex). To this end, we analysed the density of excitatory and inhibitory synapses by studying the levels of scaffolding proteins of glutamatergic and GABAergic ionotropic receptors (PSD95 and gephyrin), and the way in which their levels are correlated between functional and anatomically connected regions (parameter which we have called *synaptic coherence*). In addition, we analysed the total levels of glutamate and GABA (among other neuroamines) and the gene expression of the main subunits of ionotropic NMDA, AMPA and $GABA_A$ receptors. Given that the endocannabinoid system is the main modulator of these two systems, we analysed the gene expression of its main receptor, CB1, and the synthesis and degradation enzymes of the two main endocannabinoids, anandamide (NAPE-PLD and FAAH) and 2-arachidonylglycerol (DAGLα and MAGL). Finally, we measured the presence of perineuronal nets in the prefrontal cortex, structures of the extracellular matrix of certain GABAergic neurons associated with plastic phenomena. We were looking for common changes with the different treatments throughout the abstinence or at least equivalent changes that, even if they were not exactly the same parameter with the same time course, could tell us about the same underlying process. Despite the current view that the nucleus accumbens core and its relation to the basolateral amygdala and the prefrontal cortex are the axis on which the incubation of the seeking is directed, we did not obtain results that would lead us support such

claim. On the contrary, our results support the hypothesis that the central nucleus of the amygdala and its relationship with these glutamatergic regions orchestrate the incubation of seeking, which supports previous results published by other research groups (a summary of our results in Figure 41).

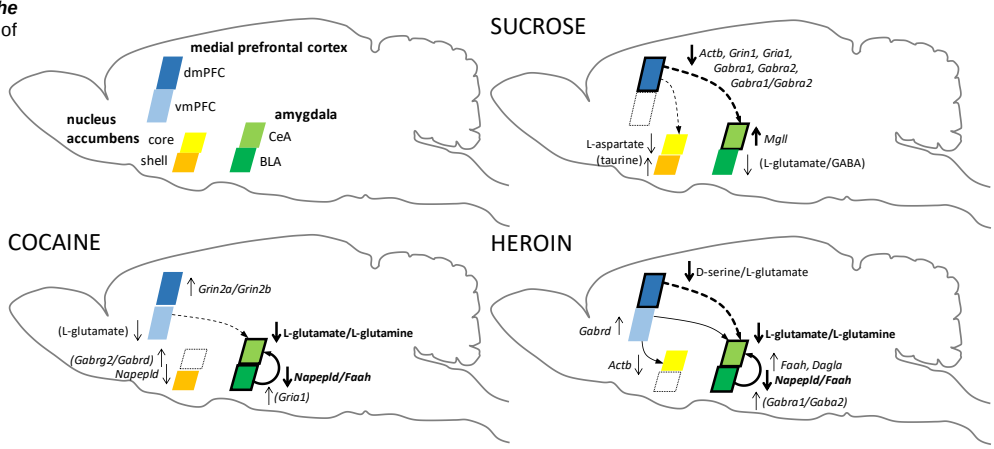


Figure 41. Summary of the main results. The results of Figures 23, 26 and 29 are presented superimposed.

4.2. THE CENTRAL NUCLEUS OF AMYGDALA: COMMON HUB IN SEEKING INCUBATION

Our main finding was that the central nucleus of the amygdala is the region around which there are equivalent, if not equal, changes during the incubation of cocaine, heroin and sucrose seeking. The paradigmatic example would be the incubation of heroin seeking. In these rats we find modifications that seem to indicate: a) an increase in the reactivity of CeA; b) increased BLA activity together with strong synaptic coherence with CeA; and c) a decrease in the plastic capacity of dmPFC and its synaptic coherence with CeA. On the other hand, the incubation of cocaine seeking would occur with processes a) and b) and the incubation of sucrose seeking with processes a) and c). We will now discuss these three patterns of effects.

a) Increased reactivity of the central nucleus of the amygdala

CeA

As discussed at the end of the Introduction (section *1.2.4. Summary of the state of the art in seeking incubation*), the central nucleus of the amygdala seems to be involved in a general way in the phenomenon of the incubation of seeking. This structure is differentially more activated during the late extinction test of cocaine, morphine, nicotine and sucrose and also its inhibition affects differentially in the cases of cocaine, methamphetamine and sucrose. In our experiments we observed changes in this region with the three substances studied. After the incubation of both heroin and cocaine we found a lower L-glutamate/L-glutamine ratio without changes in the total content of L-glutamate. This could be indicative of a faster turnover of the neurotransmitter by the astroglia of the region, affecting local phasic responses. Incubation of cocaine seeking after a self-administration protocol similar to ours is associated with an increase in NR1 levels (Lu *et al*., 2005a) in this region. We have not observed any alteration in *Grin1*, *Grin2a* or *Grin2b*, although it is true that changes in protein expression can occur without the need for changes at the level of gene expression. These results could translate into faster phasic responses but with greater sensitivity.

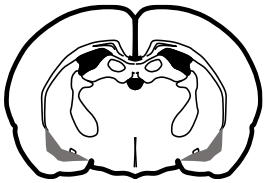
In parallel, after the consumption of sucrose, the NMDA activity in CeA increases (this is evident from the increases observed in D-serine, coagonist of these receptors) while decreasing the net excitation in a lasting manner (decreases in L-glutamate/GABA ratio). Here we find apparently antagonistic results which could be responsible for less activity in the region, but more reactivity to certain stimuli. In addition, during the incubation of sucrose seeking the levels of *Mgll* increase, the main enzyme that degrades 2-AG. In the study by Cates *et al*. (2018), using RNA-Seq after the incubation of methamphetamine seeking, the authors observe, although with little statistical power due to the low sample size, the same increases in *Mgll* in CeA. This could translate into a greater capacity to degrade the endocannabinoid 2-AG once generated and therefore a lower phasic response of it through

its CB1 receptor. The inhibition of MAGL activity in CeA provokes the decrease of anxious-like behaviours and alcohol consumption in dependent rats (Serrano *et al*., 2018) so that the increase found could have the opposite effect.

The fact that after the incubation of the seeking of sucrose and methamphetamine increases *Mgll* in CeA and that treatment with a mGluR2/3 agonist in CeA prevents the expression of the incubation of both sucrose seeking (Uejima *et al*., 2007) and cocaine (Lu *et al.*, 2007) suggests that the decrease in the L-glutamate/L-glutamine ratio after the incubation of cocaine and heroin seeking has a net effect equivalent to the increase in *Mgll*.

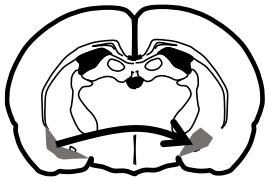
b) Increased activity of the basolateral amygdala

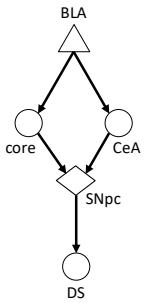
As we discussed previously, the basolateral amygdala does not seem to be involved in the expression of seeking incubation. However, it could have a role in its development. After the incubation of both cocaine and heroin seeking, we observed a lower concentration of anandamide, according to the decrease in the *Napepld*/*Faah* ratio, accompanied by increases of 2-AG in the case of heroin. These changes are preceded by changes in the glutamatergic (cocaine; and Lu *et al*., 2005a) and GABAergic systems (heroin). The decrease of anandamide and the increase of 2-AG are associated with the activation of BLA (Hill and Tasker, 2011), so that this alteration could represent a more active basal state after the incubation of the seeking. In studies with heroin addicts in prolonged abstinence, Li *et al*. (2013a) observed a greater reactivity of the amygdala before the presentation of cues related to the drug, although they do not distinguish between BLA and CeA. As the extinction tests do not activate BLA differentially between both withdrawal times but CeA, one possibility is that this increased cue-reactivity of the amygdala is located in CeA. On the other hand, after the consumption of sucrose, a decrease in the L-glutamate/GABA ratio in BLA was observed, which increased to normal during the month of abstinence. In both cases, although by different processes, this structure move from a less active state to a more active one.



... in parallel to a strong BLA-CeA synaptic coherence

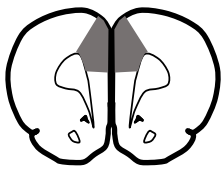
But as we mentioned before it is possible that BLA may not be playing a role in the expression of the incubation of the seeking but in its development. This region of the amygdala is involved in the early habituation of cocaine use behaviour. However, as the learning progresses, CeA takes control to maintain this memory (Murray *et al*., 2015). BLA also has the capacity to set memories in other systems under stress situations (Roozendaal *et al*., 2009). Some equivalent process could be responsible for the change of sign in the observed synaptic coherence between BLA and CeA during the incubation of cocaine and heroin seeking: one could be moving from a cue-induced seeking through BLA>core>SNpc>DS to another one in which the path CeA>SNpc>DS plays a preferential role.





c) Depletion in plasticity of the dorsomedial prefrontal cortex

The involvement of the medial prefrontal cortex in the incubation of the seeking is not clear either. In fact, it seems that it does not have a general implication but, if it did, it would be substance specific. A possible explanation for the negative results may be that this region is not necessary for the expression of the incubation of the seeking because it suffers from some hypofunction that would actually favour the increase of seeking. In the aforementioned study by Li *et al*. (2013a) the authors observe by fMRI that, after prolonged heroin abstinence, the presentation of stimuli associated with heroin had less capacity to activate the prefrontal cortex. After the consumption of both drugs of abuse, the rats of our experiments show reduced levels of *Cnr1* in dmPFC that last during the month of abstinence. During that month, the rats that consumed cocaine show decreases and increases in different subunits of AMPA and NMDA receptors and those that consumed heroin decreases in D-serine/L-glutamate ratio, reflecting a possible decrease in NMDA activity. On the other hand, rats that consumed sucrose show a strong gene depletion in dmPFC, enhanced after the month of abstinence. In addition, these rats have fewer perineuronal nets in that region (observed changes in *Gabra1*/*Gabra2* ratio could be related to the change in PNNs). Although perdurable increases in DAT have been reported in mPFC after cocaine use (Grimm *et al*., 2002), in this



study the control group is composed of rats that consumed sucrose. It is likely that such an increase is actually due to a decrease in DAT in the mPFC of the rats that consumed sucrose. In fact, other authors have not been able to see changes in DAT after cocaine abstinence (Ben-Shahar *et al*., 2006). Therefore, it is possible that at least the incubation of heroin and especially sucrose seeking will lead to a hypofunction of dmPFC.

… in parallel to a depleted dmPFC-CeA synaptic coherence

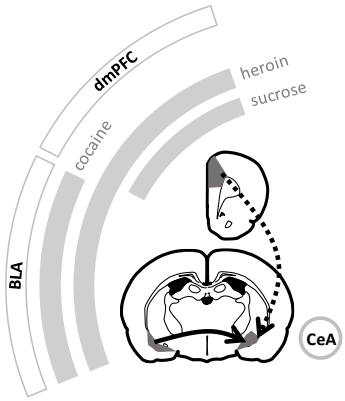


Figure 42. Proposed model based on the results of the 2nd goal.

Perhaps such a hypofunction explains why in people undergoing diets in which it is not possible to reduce craving, there is less control of dlPFC over the amygdala when food-related stimuli are presented to the probands (Kahathuduwa *et al*., 2018) . In addition, this hypofunction could be responsible for the loss of synaptic coherence between dmPFC and CeA observed after the incubation of heroin and sucrose seeking. Since the lack of cortical inhibition has been postulated as one of the causes of addiction (Everitt and Robbins, 2016), such a proposal fits quite well with contemporary literature.

Therefore, and depending on the paradigm and/or substance studied and/or the level of incubation reached, after prolonged abstinence we find a central nucleus of the amygdala especially reactive to the cues associated with consumption, and at the same time (Figure 42):
- a basolateral amygdala with increased basal activity; and/or
- a dorsomedial prefrontal cortex incapable of inhibiting CeA responses.

4.3. CHANGES IN THE NUCLEUS ACCUMBENS DURING THE INCUBATION OF SEEKING

As we discussed in the Introduction, the nucleus accumbens is undoubtedly the region that has received the most attention in the literature on incubation of seeking. However, almost all studies have been conducted with cocaine and those who have used other non-psychostimulant substances have not measured the same parameters. Its core region appears involved in the incubation of psychostimulants seeking but it does not seem to be involved in incubation of seeking of a natural reinforcer such as sucrose. We did not find any differential change in the rats that incubated cocaine seeking compared to those of early abstinence. The only changes we have seen appear on the first day of abstinence and last up to following month. There are increases in the levels of L-glutamate and L-aspartate, possibly due to decreases in GLT1 transporter levels (Fischer-Smith *et al*., 2012). In the shell found increases in the *Gria1*/*Gria2* ratio (which could result in increases in CP-AMPAR), decreases in *Napepld*/*Faah*, and may be the establishment of a BLA>shell synaptic coherence, all of them lasting effects. We also found effects related to abstinence per se, namely increases of the ratio *Gabrg2*/*Gabrd* after consumption that normalizes after the month of abstinence during which the levels of *Napepld* descend.

The use of heroin, meanwhile, led to declines in the expression of *Actb* and increases in its synaptic coherence with dmPFC during the month of abstinence. This last result is reminiscent of that observed by Luís *et al*. (2017) after the use of cocaine, in which they found a strengthening of this path after the consumption that lasted during the abstinence (they argue that this connection increases throughout the abstinence, but they do not prove it statistically). Furthermore, in the shell there were perdurable increases in the levels of *Grin2a*, *Grin2b*, *Gria1* and *Actb*.

The major perdurable changes related to core were after the consumption of sucrose: increases in the ratios L-glutamate/GABA and PSD95/gephyrin accompanied by decreases in *Gria2* and *Napepld* associated with GABAergic synapses (as evidenced by the decrease in variable F'13 of the PCA, composed of these two genes and the four subunits of $GABA_A$ studied). That is, after sucrose consumption, the nucleus accumbens core undergoes a GABAergic depletion in favour of glutamatergic. This change could be related to the synaptic coherence observed between vmPFC and core after the consumption of sucrose. The only changes observed after sucrose seeking incubation were decreases in L-aspartate levels and the disappearance of synaptic coherence with dmPFC, as was the case with CeA. In the shell region we observed taurine increases only after a day of

abstinence, and lasting changes in the levels of *Grin1*, *Grin2a*/*Grin2b*, *Gabrg2* and *Gabrg2*/*Gabrd* (decreases) and of *Gabrd*, *Cnr1* and *Faah* (increases). In summary, our results seem to indicate that the core region presents a greater activity in a lasting way during abstinence, at least after the consumption of cocaine and, especially, of sucrose. In addition, it seems that the shell region shows greater activity, also in a lasting way, after the consumption of the drugs of abuse but lower after sucrose.

It is not at all surprising that we have not found similar changes in the three substances studied. In the literature on the subject we find shell and core manipulations at short or long abstinence times capable of modifying the response rate in animals (Conrad *et al*., 2008; Lee *et al*., 2013; Li *et al*., 2013b; Loweth *et al*., 2014; Wang *et al*., 2018). However, by studying the incubation of sucrose seeking, the injection of a D1 receptor inhibitor in core or shell reduced the responses equally to both withdrawal times (Grimm *et al*., 2011). It has been shown that cocaine seeking incubation occurs with increases in CP-AMPAR levels in both core and shell (Conrad *et al*., 2008), although the origin of these synapses is uncertain (Ma *et al*., 2014), and the same happens with methamphetamine (Scheyer *et al*., 2016), another psychostimulant. However, by studying the incubation of seeking of natural reinforcers we find disparate results. Studies using food as reinforcer have shown increases in AMPAR/NMDAR ratio, as with cocaine, induced by CP-AMPAR increases in the case of normal animal food but by CI-AMPAR in the case of fatty food (Dingess *et al*., 2017). Furthermore, decreases are observed in this ratio when sucrose was the reinforcer studied (Counotte *et al*., 2014).

Therefore, we believe that the changes observed in the experiments studying psychostimulants are only relevant to the incubation of the seeking of such substances or even more, that they are effects of prolonged abstinence in pathways related to the seeking but that are not related to the incubation phenomenon.

3rd Goal: CHANGES IN STRESS RELATED PARAMETERS DURING SEEKING INCUBATION

During the sacrifice and dissection of the rats we noticed that the adrenal glands of some of them were larger than normal. Therefore, in addition to studying parameters related to glutamatergic and GABAergic transmission, we decided to analyse parameters related to stress, given the relationship between stress and substance use, and given that its relationship with the incubation of seeking is not clear. We focused on those variables for which we had data from all the experimental groups, such as the weight of the adrenal glands, the plasma levels of corticosterone and several amines (taking advantage of the capillary electrophoresis technique) and the levels of expression of adrenergic receptors. It is noteworthy that certain brain areas (including some of the ones that we have studied) regulate the stress response. In addition, the endocannabinoid system, analysed in the previous goal, also regulate stress-related procesess. For these reasons, in this section we will return to discuss some results mentioned in the previous one, this time from the point of view of stress.

4.4. ACTIVITY OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

In drug users there is an affectation of stress systems, understood in its broadest sense. For example, in cocaine and alcohol users, stress and the cues related to consumption are able to induce craving and anxiety and activate the autonomic nervous system (ANS) and the hypothalamic-pituitary-adrenal (HPA) axis. Moreover, these changes are greater in the case of high frequency consumers (Fox *et al*., 2005). It has been observed in both humans and laboratory animals that drug consumption alters the activity of the ANS and the HPA axis itself (Goldfarb and Sinha, 2018; Ulrich-Lai and Herman, 2009). Heroin consumption causes inactivation of the HPA axis, while cocaine use activates it. Moreover, abstinence from both substances causes hyperactivity in the HPA axis in consumers (Brown *et al*., 2006). In addition, rats that consume cocaine following extended access protocols have higher levels of plasma corticosterone than those with restricted access, probably

because increased exposure to the drug leads to greater activation of the HPA axis (Mantsch *et al*., 2003; Figure 43). In parallel, drug users have a dysfunctional HPA axis, with higher levels of plasma cortisol and higher plasma responses (Goldfarb and Sinha, 2018).

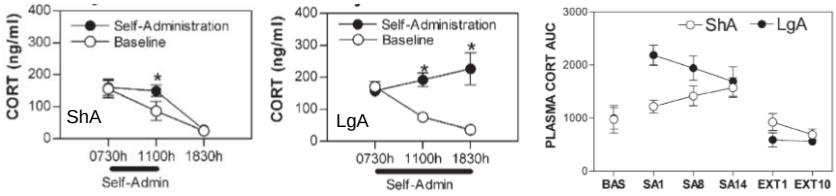


Figure 43. Daily levels of plasma corticosterone. The plasma levels of corticosterone measured at three times during the days of self-administration with restricted access (ShA, *left*) or extended (LgA, *center*) to cocaine, as well as the evolution of the area under the curve (AUC) of this parameter throughout the sessions (*right*). (Mantsch *et al*., 2003).

On the contrary, the consumption of natural reinforcers, when contingently presented, decreases the level of activation of the ANS and the HPA axis, unless the cues associated with its consumption are presented in the absence of the reinforcer: in this case they are also activated (Ulrich-Lai and Herman, 2009). In some studies on craving incubation in humans, parameters related to the activation of ANS induced by cues and baseline anxiety of the subjects are measured (Figure 3). It is observed that this activation of the ANS (cardiovascular stress) does not present the same kinetics as the incubation of craving methamphetamine, heroin, tobacco or alcohol. On the other hand, baseline anxiety experiences a parallel incubation in the case of methamphetamine but this effect is not observed in the case of heroin or alcohol. None of the studies measures changes in cue-induced anxiety.

In studies of incubation of seeking carried out with rats, increases in plasma corticosterone are observed during the early abstinence of cocaine that normalizes three weeks later (Thiel *et al*., 2012). When the substance used is sucrose, no change is observed (Grimm *et al*., 2016). However, in this study the rats are anesthetized before blood collection so it is possible that any effect, if present, could be blunted by the anesthesia. In our rats we observed an adrenal hyperplasia after self-administration sessions of both drugs of abuse, not always accompanied by increases in plasma corticosterone. This may be due to the fact that the effect is not consistent or that there was a lot of variability in the morning time at which the animals were sacrificed.The rats that consumed sucrose, on the contrary, did not suffered changes in the size of the adrenal glands. However, plasma corticosterone levels were altered. After a day of abstinence their average levels were below the mean level of the control group and above the controls after a month of abstinence. Therefore, we can conclude that the use of cocaine and heroin strongly activated the HPA axis, leading to a hypertrophy of the adrenal glands. Conversely, sucrose abstinence occurred concomitantly with an increase in HPA axis activation.

4.5. STRESS RELATED CENTRAL CHANGES

a) Equivalences with chronic stress in the expression of eCB system

Adrenomegaly is usually found in rats subjected to chronic stress (Ulrich-Lai and Herman, 2009). These protocols, as well as the administration of corticosterone, are also capable of inducing changes in the central endocannabinoid system (Bowles *et al*., 2012; Gray *et al*., 2016; Häring *et al*., 2012; McLaughlin and Gobbi, 2012; Ramikie and Patel, 2012). The general effect is usually decreases in anandamide and increases in 2-AG. For example, in BLA the above-mentioned manipulations can cause long-lasting decreases in anandamide tone due to increases in FAAH, its main degradation enzyme, and increased 2-AG responses that return to baseline (Ramikie and Patel, 2012). The rats in our experiments present lasting changes in the eCB system that are observed on the first day of abstinence and last at least until the month of abstinence. The consumption of both drugs of abuse resulted in dmPFC declines in *Cnr1*. In addition, cocaine consumption induced decreases in *Napepld*/*Faah* in shell and heroin increases in *Faah* in the vmPFC. This is compatible with potential decreases in the levels of AEA. Surprisingly, the consumption of sucrose also caused changes in the same direction: in dmPFC increases in *Dagla*/*Mgll*, in the core decreases in *Napepld* and in the shell increases in *Faah* and *Cnr1*. Consequently, we may suggest that there could be increases in 2-AG and decreases in AEA.

It is not clear if the changes observed in the endocannabinoid system are partially or totally related to the activity (increased or decreased) of the HPA axis. However, due to the direction of the changes, this axis is a good candidate.

b) Involvement of the eCB system in stress and anxiety

Regardless of whether the changes in the eCB system derive from the activity of the HPA axis or not, this is not the only relationship between the two elements. Indeed, just as the HPA axis is capable of regulating the expression of the eCB system, this system is also capable of modulating stress and anxiety (Häring *et al*., 2012; Hill and Tasker, 2011). Anandamide in the basolateral amygdala has been postulated to be the *gatekeeper* of HPA axis activation (Hill and Tasker, 2012). An increased activity of FAAH, leading to decreased levels of the endocannabinoid, is necessary for the HPA axis to be activated. On the other hand, MAGL activity in CeA has anxiogenic properties (Serrano et al., 2018). In addition, the activation of CB1 in both regions of the amygdala has antagonistic effects on anxiety (McLaughlin and Gobbi, 2012). Moreover, the interaction between the CB1 receptor of the prefrontal cortex and corticosterone is one of the mechanisms to terminate such activation (Ulrich-Lai and Herman, 2009, Hill and Tasker, 2012). Therefore, the effects on the eCB system observed after the consumption of the different substances in the amygdala and in the prefrontal cortex could be altering the ability of the animals to control behaviour under stress and/or anxiety (Figure 44).

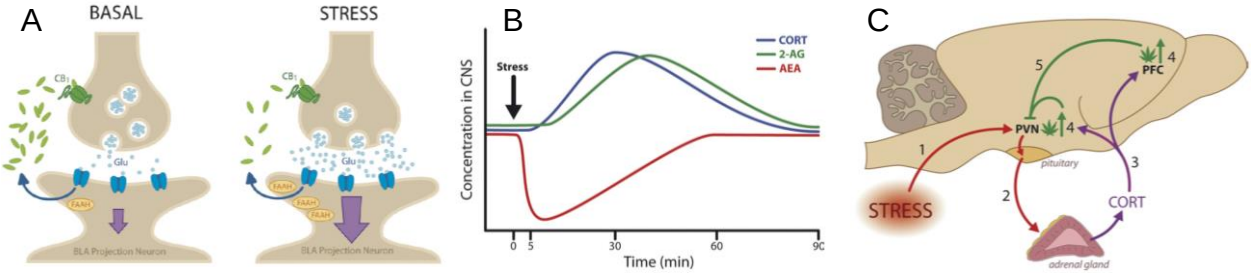


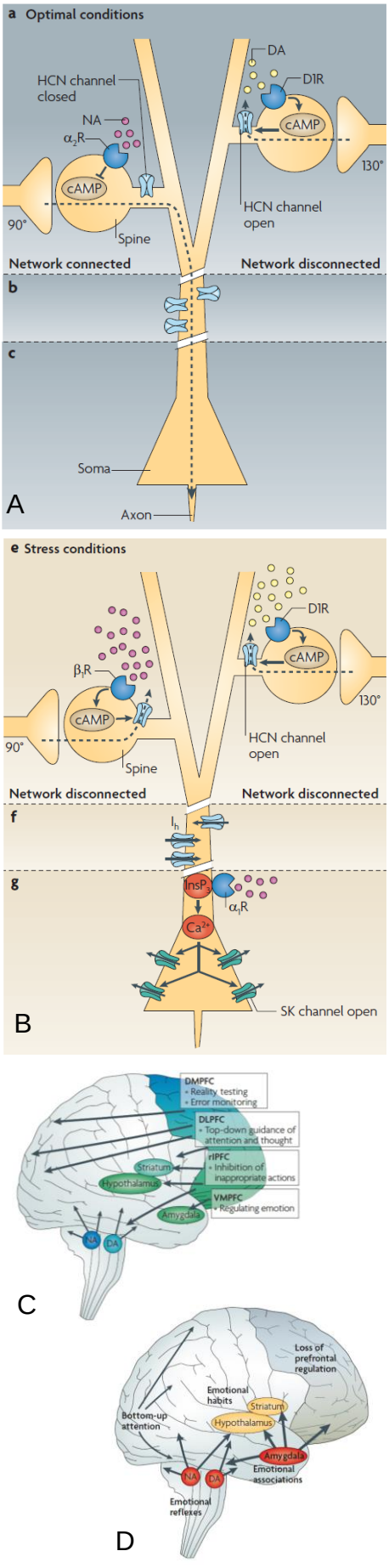
Figure 44. Activation of the HPA axis by the basolateral amygdala. In situations of stress, FAAH activity (A) is induced, causing anandamide levels to decrease in BLA, which promotes activation of the HPA axis (B). The subsequent rise of corticosterone will promote the termination of the signal thanks, among other mechanisms, to its interaction with the endocannabinoid system in the PFC (C) (Hill and Tasker, 2012).

Found two patterns in our experiments: the one observed after the consumption of cocaine and heroin and the one observed after the consumption of sucrose. Firstly, the intake of both drugs of abuse caused decreases of *Cnr1* in dmPFC while prolonged abstinence decreases the *Napepld*/*Faah* ratio in BLA. This could translate into a lower capacity of the cortex to terminate the stress signal (through the corticosterone-CB1 interaction) for a prolonged period accompanied, after one month of abstinence, by a greater capacity of BLA to activate the HPA axis (due to an increased anandamide degradation capacity).

In addition, the sucrose intake caused increases in the *Dagla*/*Mgll* ratio in dmPFC accompanied, after one month of abstinence, by increased *Mgll* in CeA. In contrast to drugs of abuse, an increase in the ability of dmPFC to synthesize 2-AG would give rats greater cortical control over the termination of the stress signal. This may explain why they have somewhat lower levels of corticosterone after a day of abstinence. However, after one month of abstinence the decreases in 2-AG in CeA could render them more anxious.

c) Changes in noradrenaline reactivity in BLA-dmPFC

Another effect of chronic stress, which affects the structures mentioned in the previous section, is the amygdala hyperresponsiveness and the hyporesponsiveness of the prefrontal cortex (Roozendaal *et al*., 2009). This is not surprising if we consider how activity is regulated in situations of stress, when noradrenaline (NA) levels increase momentarily. Both regions, BLA and PFC, receive NA from the locus coeruleus, unlike the nucleus accumbens shell and CeA that receive NA from the nucleus of the solitary tract (Kvetnansky *et al*., 2009). Both regions express NA receptors of high ($\alpha 2$) and low affinity ($\alpha 1$, $\beta 1$), which will be activated depending on the concentration of NA. In normal situations only those with high affinity ($\alpha 2$)



will be active. However, when the NA concentration increases, those with low affinity will enter into action (α1, β1). In the prefrontal cortex, the activation of α2 receptors in normal situations allows a correct functioning of the region, but when the α/β1 receptors are activated in situations of stress this region is silenced (Arnsten, 2009). On the contrary, in BLA, these receptors have opposite effects to the ones they have in the prefrontal cortex, so in stress situations this region will be overactive (Roozendaal *et al*., 2009). Therefore, the relative expression of these receptors (especially those of low affinity) in BLA and PFC can have consequences on the activity of these regions during high arousal situations (Figure 45).

After the consumption of the substances studied in this Thesis, the rats suffered different changes in the levels of expression of *Adra1*, *Adra2a* and *Adrb1*, depending on the substance consumed and the time of abstinence. Only sucrose intake caused perdurable changes in the expression of these genes. These changes consisted on decreases in the levels of the three genes in dmPFC, in accordance with the generalized gene depletion observed in this region. In addition, after the month of abstinence, the depletion of *Adrb1* was even greater, (along with an increase in BLA of the same gene). Therefore, although the ability of norepinephrine to inactivate dmPFC potentially decreased (as also decreased the ability to activate this region in normal situations), it did so by increasing in parallel its ability to activate BLA. After the incubation of heroin seeking we found increases in *Adrb1* in BLA, possibly enhancing the ability of the amygdala to become active during stressful situations. Finally, after the incubation of cocaine seeking we observed that the levels of *Adrb1* in BLA and dmPFC are related in such a way that the higher their rise in BLA (and therefore their ability to be activated) the lower their levels in dmPFC (and therefore, its capacity to be inactivated) and vice versa.

It seems, therefore, that, with the exception of rats that consumed heroin, the increases in *Adrb1* in BLA are accompanied by changes of *Adrb1* in dmPFC in the opposite direction, although it is true that in the case of sucrose abstinence they go accompanied by a general hypofunction of dmPFC. This hypofunction, as we saw, was accompanied by a depletion in the density of perineuronal nets. Recently, it has been reported that subjecting adolescent rats to chronic stress produces, as well as that observed after the consumption of sucrose, decreases in plasma corticosterone levels and in perineuronal nets in dmPFC (de Araújo Costa Folha *et al*., 2017).

In spite of the fact that in the previous section we concluded that BLA does not seem necessary for the expression of the incubation of seeking, the changes in the expression of the β1 receptor in BLA are interesting since they open the possibility that in the presence of elevated noradrenaline increased activation of BLA is able to eclipse the pharmacological inhibitions that are targeted to this area (Morena *et al*., 2016a). As it happens in experiments on seeking performed in NAc, we can find different effects depending on the pharmacological tool that is used to inactivate a region (Yun *et al*., 2004). In fact, the late positive potential (LPP), used by Parvaz *et al*. (2016) as a neurophysiological record of craving in abstinent cocaine addicts when studying craving incubation in humans, seems to depend on the activation of the β1 receptors of the basolateral amygdala (de Rover *et al*., 2012).

Figure 45. Effects of noradrenaline on the activity of the prefrontal cortex. In normal situations, the prefrontal cortex functions normally (C) because only the high affinity noradrenaline receptors (A) are active. In situations of stress, it loses activity in favour of the amygdala (D) when low affinity receptors are activated (B) (Arnsten, 2009).

4.6. ADDITIONAL OBSERVED CHANGES

Other aspects not directly related to the incubation of cue-induced seeking will now be succinctly discussed.

a) Escalation

One of the differences between the protocols of restricted access and extended access is that the latter promote the escalation in the consumption of drugs of abuse (Ahmed *et al*., 2000; Ahmed and Koob, 1999). They also differ in that extended access leads to greater activation of the HPA axis (Mantsch *et al*., 2003), and may even cause adrenal hyperplasia, as in our case. Given that stress favours the formation of habits (Everitt and Robbins, 2016), it would be plausible that such escalation was due to the degree of exposure to corticosterone suffered by these rats. In fact, the administration of corticosterone in the dorsolateral striatum, involved in the formation of habits, promotes them, something that does not happen if it is administered in the dorsomedial region (Siller-Pérez *et al*., 2017). The authors point to the interaction between corticosterone and the CB1 receptor as a possible mechanism, since their levels are much higher in the lateral part of the striatum than in the medial part (Van Waes *et al*., 2012; Figure 46). However, it has been recently suggested that the addictive phenotype, including escalation, does not depend on the formation of habits since it can be induced with protocols that, even if involving extended access, prevent their formation (Singer *et al*., 2017). Therefore, although habit formation is not necessary for the escalation, it is possible that in the protocols of extended access habit-formation is facilitated.

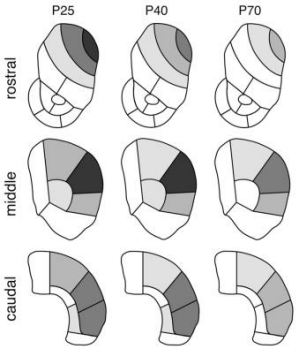


Figure 46. Expression of CB1 receptor in rat dorsal striatum. The shades of gray indicate the areas of highest density of expression of the CB1 receptor, measured in the rostral, medial and caudal striatum, in rats of 25, 40 and 70 days of life (Van Waes *et al*., 2012).

b) Anhedonia

People addicted to psychostimulants usually suffer from anhedonia (Leventhal *et al*., 2010), a fact that has been reproduced in laboratory animals under extended access paradigms (Mantsch *et al*., 2014). Animals subjected to stress also experiment this condition. In these animals, the administration of FAAH inhibitors or CB1 agonists prevents anhedonia (Morena *et al*., 2016b). In humans, anhedonia is negatively correlated to the activity of several limbic areas, including the nucleus accumbens (Keller *et al*., 2013). Studies on anhedonia in animals focusing in this region have shown that the concentration of shell anandamide is related to the hedonic pleasure of natural reinforcers (Mahler *et al*., 2007; Shinohara *et al*., 2009). Therefore, the decrease in shell of the *Napepld*/*Faah* ratio experimented by the rats that consumed cocaine could result in a reduction of anandamide in the region and be responsible for the expected anhedonia in these rats. In fact, it is already been suggested that rats with extended access to cocaine, unlike those with restricted access, have decreased basal anandamide levels (trend, Figure 47) (Orio *et al*., 2009). After one month, the levels of *Napepld* fall further so it is possible that prolonged abstinence potentiates anhedonia. As for the natural reinforcers, the capacity of these to evoke dopamine increases in shell habituated quickly. Since THC, through CB1, is able to provoke again those dopamine increases in shell with a previously used (De Luca *et al*., 2012), the changes observed in the eCB system in shell after sucrose intake (increases of *Faah* and *Cnr1*) could be reponsible for this habituation.

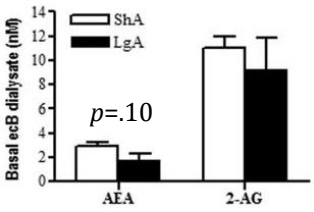


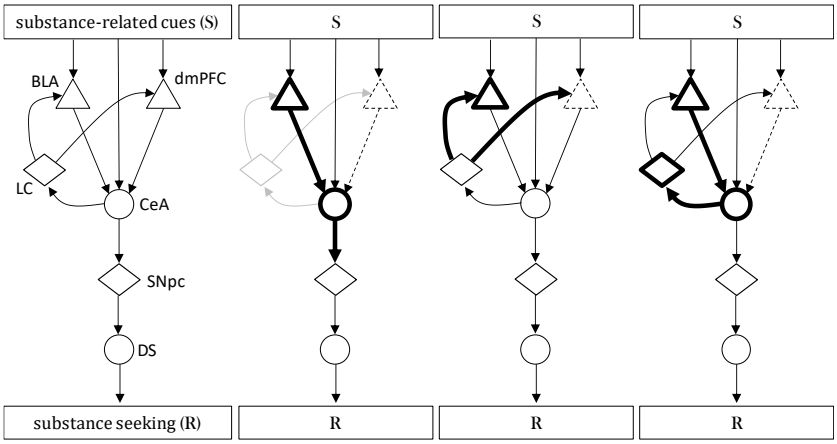
Figure 47. AEA and 2-AG basal levels in shell. The extended access to cocaine (LgA) caused a downward trend in the levels of AEA compared to rats with restricted access to cocaine (ShA) (Orio *et al*., 2009).

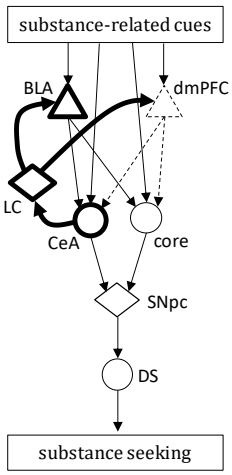
c) Stress-induced seeking incubation

Even if BLA is not involved in the expression of cue-induced seeking incubation, it is possible that it has a role in the incubation of stress-induced seeking. As we saw in the Introduction, stress-induced seeking incubates in the case of heroin (Shalev *et al*., 2001) but not methamphetamine (Shepard *et al*., 2004). In addition, voluntary abstinence promoted by giving the rats the choice to consume food, which in itself should be anxiolytic, prevents the expression of seeking induced by methamphetamine cues (Caprioli *et al*., 2017) but not heroin (Venniro *et al*., 2017). It seems, therefore, that in the case of heroin, the stress response is enhanced after a month of abstinence. This could be related to the increase in *Faah* and *Adrb1* that we have observed in the BLA.

PROPOSED MECHANISM OF SEEKING INCUBATION

Depending on the specific approach, several mechanisms could be linked to the incubation of seeking. First, we suggest a situation with a hyperreactivity of the central nucleus of the amygdala to cues together with a basolateral amygdala also more reactive and/or a dysfunctional dorsomedial prefrontal cortex. There could also be a role for a more reactive basolateral amygdala and/or a less reactive or dysfunctional medial prefrontal cortex in high arousal situations, when noradrenaline increases. Finally, a more 'anxiogenic' central nucleus of the amygdala together with a basolateral amygdala of a similar profile and/or a dorsomedial prefrontal cortex activity associated to decrease anxiety could also be suggested to operate in the phenomenon. Separately, any of the three possible interpretations could be responsible for the phenomenon of seeking incubation. An interesting question is: but, among all these proposed mechanisms, what is the most likely?





We propose an explanation that focuses on the central nucleus of the amygdala, and that gives importance to noradrenaline as a key piece in relapse during prolonged abstinence. Importantly, the involved NA would not be the one released in the extended amygdala (Smith and Aston-Jones, 2008) but the noradrenaline release induced by CeA. During the self-administration sessions the basolateral amygdala could promote the association between the cues and the reinforcer obtained, as well as associated procedural memories, helped by the activation of the HPA axis (Roozendaal and McGaugh, 2011). By bringing the animals back to the operant conditioning boxes, the contextual clues promote an arousal state as a consequence of a heightened cue-reactivity of the CeA, aided by a BLA that is also more reactive and/or by a blunted activity of the dmPFC. In this new state, with the release of noradrenaline by the locus coeruleus (LC) induced by CeA (Bouret *et al*., 2003) to take advantage of the situation predicted by contextual cues, the behaviour will be guided by the CeA>SNpc>DS and/or BLA>core>SNpc>DS pathways, according to the protocol used and the sensitivity of BLA to norepinephrine. This state of alert will last longer, increasing the number of unsuccessful attempts during extinction test, due to a dmPFC with less capacity to stop it and to extinguish the behaviour.

In a paradigm of CPP, by bringing the animals back to the pair of chambers, the alert state will promote visits and more time spent at chamber where the reinforcer was received. In a protocol of locomotor sensitization the key could be the injection itself, which the animal associates with the effects of the drug. Thus, the locomotor sensitization will be increased thanks to the arousal state induced by CeA. Finally, in a paradigm of incubation of conditioned fear, the cues associated with punishment will also provoke a state of alert that will enhance the fear response.

CONCLUSIONS

1. We have not found behavioural variables during the self-administration sessions that predicted the degree of incubation of seeking.

2. We have not found any biochemical variable that is equally affected during the incubation of the seeking of the three substances.

3. Among the parameters related to the glutamatergic, GABAergic and endocannabinoid systems, which showed changes during the incubation of seeking, we found equivalences between the three substances in the central nucleus of the amygdala, after one month of abstinence:

a) Decreases of L-glutamate/L-glutamine after one month of withdrawal of cocaine and heroin;

b) *Mgll* increases after one month of withdrawal of sucrose.

4. We also find, after the month of abstinence, equivalences between pairs of substances:

a) *Napepld*/*Faah* ratio decreases in BLA and a positive synaptic coherence appears between BLA and CeA after one month of cocaine and heroin withdrawal;

b) Lack of synaptic coherence between dmPFC and CeA and decreases in NMDAR transmission in dmPFC and after one month of heroin (D-serine/L-glutamate) and sucrose (*Grin1*) withdrawal.

5 Among the parameters related to stress we find:

a) Adrenal hyperplasia after the consumption of cocaine and heroin and plasma corticosterone increases during the incubation of sucrose seeking;

b) Differential changes in the levels of *Adrb1* in BLA and dmPFC.

6. In addition, the consumption of sucrose caused a depletion of perineuronal nets in dmPFC accompanied by a more acute general gene depletion after the incubation of the seeking.

REFERENCES

Abdolahi, A., Acosta, G., Breslin, F.J., Hemby, S.E., Lynch, W.J., 2010. Incubation of nicotine seeking is associated with enhanced protein kinase A-regulated signaling of dopamine- and cAMP-regulated phosphoprotein of 32 kDa in the insular cortex. Eur. J. Neurosci. https://doi.org/10.1111/j.1460-9568.2010.07114.x

Adhikary, S., Caprioli, D., Venniro, M., Kallenberger, P., Shaham, Y., Bossert, J.M., 2017. Incubation of extinction responding and cue-induced reinstatement, but not context- or drug priming-induced reinstatement, after withdrawal from methamphetamine. Addict. Biol. https://doi.org/10.1111/adb.12386

Ahmed, S.H., Koob, G.F., 1999. Long-lasting increase in the set point for cocaine self- administration after escalation in rats. Psychopharmacology (Berl). 146, 303–312. https://doi.org/10.1007/s002130051121

Ahmed, S.H., Walker, J.R., Koob, G.F., 2000. Persistent increase in the motivation to take heroin in rats with a history of drug escalation. Neuropsychopharmacology 22, 413–421. https://doi.org/10.1016/S0893-133X(99)00133-5

Airavaara, M., Pickens, C.L., Stern, A.L., Wihbey, K.A., Harvey, B.K., Bossert, J.M., Liu, Q.R., Hoffer, B.J., Shaham, Y., 2011. Endogenous GDNF in ventral tegmental area and nucleus accumbens does not play a role in the incubation of heroin craving. Addict. Biol. https://doi.org/10.1111/j.1369-1600.2010.00281.x

American Psychiatric Association, 2013. Diagnostic and statistical manual of mental disorders : DSM-5. American Psychiatric Association, DSM. https://doi.org/10.1176/appi.books.9780890425596.744053

Aoyama, K., Barnes, J., Grimm, J.W., 2014. Incubation of saccharin craving and within-session changes in responding for a cue previously associated with saccharin. Appetite. https://doi.org/10.1016/j.appet.2013.10.003

Arnsten, A.F.T., 2009. Stress signalling pathways that impair prefrontal cortex structure and function. Nat. Rev. Neurosci. https://doi.org/10.1038/nrn2648

Batra, P., Das, S.K., Salinardi, T., Robinson, L., Saltzman, E., Scott, T., Pittas, A.G., Roberts, S.B., 2013. Relationship of cravings with weight loss and hunger. Results from a 6month worksite weight loss intervention. Appetite. https://doi.org/10.1016/j.appet.2013.05.002

Bedi, G., Preston, K.L., Epstein, D.H., Heishman, S.J., Marrone, G.F., Shaham, Y., De Wit, H., 2011. Incubation of cue-induced cigarette craving during abstinence in human smokers. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2010.07.014

Belin, D., Balado, E., Piazza, P.V., Deroche-Gamonet, V., 2009. Pattern of intake and drug craving predict the development of cocaine addiction-like behavior in rats. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2008.05.031

Ben-Shahar, O., Keeley, P., Cook, M., Brake, W., Joyce, M., Nyffeler, M., Heston, R., Ettenberg, A., 2007. Changes in levels of D1, D2, or NMDA receptors during withdrawal from brief or extended daily access to IV cocaine. Brain Res. https://doi.org/10.1016/j.brainres.2006.10.069

Ben-Shahar, O., Moscarello, J.M., Ettenberg, A., 2006. One hour, but not six hours, of daily access to self-administered cocaine results in elevated levels of the dopamine transporter. Brain Res. 1095, 148–153. https://doi.org/10.1016/j.brainres.2006.04.002

Ben-Shahar, O., Obara, I., Ary, A.W., Ma, N., Mangiardi, M.A., Medina, R.L., Szumlinski, K.K., 2009. Extended daily access to cocaine results in distinct alterations in Homer 1b/c and NMDA receptor subunit expression within the medial prefrontal cortex. Synapse 63, 598–609. https://doi.org/10.1002/syn.20640

Ben-Shahar, O., Sacramento, A.D., Miller, B.W., Webb, S.M., Wroten, M.G., Silva, H.E., Caruana, A.L., Gordon, E.J., Ploense, K.L., Ditzhazy, J., Kippin, T.E., Szumlinski, K.K., 2013. Deficits in Ventromedial Prefrontal Cortex Group 1 Metabotropic Glutamate Receptor Function Mediate Resistance to Extinction during Protracted Withdrawal from an Extensive History of Cocaine Self-Administration. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.3710-12.2013

Bienkowski, P., Rogowski, A., Korkosz, A., Mierzejewski, P., Radwanska, K., Kaczmarek, L., Bogucka-Bonikowska, A., Kostowski, W., 2004. Time-dependent changes in alcohol-seeking behaviour during abstinence. Eur. Neuropsychopharmacol. https://doi.org/10.1016/j.euroneuro.2003.10.005

Blackwood, C.A., Hoerle, R., Leary, M., Schroeder, J., Job, M.O., McCoy, M.T., Ladenheim, B., Jayanthi, S., Cadet, J.L., 2018. Molecular Adaptations in the Rat Dorsal Striatum and Hippocampus Following Abstinence-Induced Incubation of Drug Seeking After Escalated Oxycodone Self-Administration. Mol. Neurobiol. https://doi.org/10.1007/s12035-018-1318-z

Boswell, R.G., Kober, H., 2016. Food cue reactivity and craving predict eating and weight gain: A meta-analytic review. Obes. Rev. https://doi.org/10.1111/obr.12354

Boucard, A., Marchand, A., Noguès, X., 2007. Reliability and validity of structural equation modeling applied to neuroimaging data: A simulation study. J. Neurosci. Methods 166, 278–292. https://doi.org/10.1016/j.jneumeth.2007.07.011

Bouret, S., Duvel, A., Onat, S., Sara, S.J., 2003. Phasic activation of locus ceruleus neurons by the central nucleus of the amygdala. J. Neurosci. 23, 3491–7.

Bowles, N.P., Hill, M.N., Bhagat, S.M., Karatsoreos, I.N., Hillard, C.J., McEwen, B.S., 2012. Chronic, noninvasive glucocorticoid administration suppresses limbic endocannabinoid signaling in mice. Neuroscience. https://doi.org/10.1016/j.neuroscience.2011.08.048

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254. https://doi.org/10.1016/0003-2697(76)90527-3

Brecht, M.L., Herbeck, D., 2014. Time to relapse following treatment for methamphetamine use: A long-term perspective on patterns and predictors. Drug Alcohol Depend. https://doi.org/10.1016/j.drugalcdep.2014.02.702

Brown, T.T., Wisniewski, A.B., Dobs, A.S., 2006. Gonadal and Adrenal Abnormalities in Drug Users: Cause or Consequence of Drug Use Behavior and Poor Health Outcomes. Am. J. Infect. Dis. 2, 130–135.

Burokas, A., Martín-García, E., Espinosa-Carrasco, J., Erb, I., McDonald, J., Notredame, C., Dierssen, M., Maldonado, R., 2018. Extinction and reinstatement of an operant responding maintained by food in different models of obesity. Addict. Biol. 23, 544–555. https://doi.org/10.1111/adb.12597

Calabrese, E.J., 2016. Preconditioning is hormesis part I: Documentation, dose-response features and mechanistic foundations. Pharmacol. Res. https://doi.org/10.1016/j.phrs.2015.12.021

Calu, D.J., Stalnaker, T.A., Franz, T.M., Singh, T., Shaham, Y., Schoenbaum, G., 2007. Withdrawal from cocaine self-administration produces long-lasting deficits in orbitofrontal-dependent reversal learning in rats. Learn. Mem. https://doi.org/10.1101/lm.534807

Cannella, N., Oliveira, A.M.M., Hemstedt, T., Lissek, T., Buechler, E., Bading, H., Spanagel, R., 2018. Dnmt3a2 in the Nucleus Accumbens Shell Is Required for Reinstatement of Cocaine Seeking. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.0600-18.2018

Caprioli, D., Venniro, M., Zeric, T., Li, X., Adhikary, S., Madangopal, R., Marchant, N.J., Lucantonio, F., Schoenbaum, G., Bossert, J.M., Shaham, Y., 2015a. Effect of the novel positive allosteric modulator of metabotropic glutamate receptor 2 AZD8529 on incubation of methamphetamine craving after prolonged voluntary abstinence in a rat model. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2015.02.018

Caprioli, D., Venniro, M., Zhang, M., Bossert, J.M., Warren, B.L., Hope, B.T., Shaham, Y., 2017. Role of Dorsomedial Striatum Neuronal Ensembles in Incubation of Methamphetamine Craving after Voluntary Abstinence. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.3091-16.2017

Caprioli, D., Zeric, T., Thorndike, E.B., Venniro, M., 2015b. Persistent palatable food preference in rats with a history of limited and extended access to methamphetamine self-administration. Addict. Biol. 20, 913–26. https://doi.org/10.1111/adb.12220

Cates, H.M., Li, X., Purushothaman, I., Kennedy, P.J., Shen, L., Shaham, Y., Nestler, E.J., 2018. Genome-wide transcriptional profiling of central amygdala and orbitofrontal cortex during incubation of methamphetamine craving. Neuropsychopharmacology 43, 2426–2434. https://doi.org/10.1038/s41386-018-0158-x

Chauvet, C., Goldberg, S.R., Jaber, M., Solinas, M., 2012. Effects of environmental enrichment on the incubation of cocaine craving. https://doi.org/10.1016/j.neuropharm.2012.05.014

Chen, B., Wang, Y., Liu, X., Liu, Z., Dong, Y., Huang, Y.H., 2015. Sleep Regulates Incubation of Cocaine Craving. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.1065-15.2015

Chomczynski, P., Sacchi, N., 2006. The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: Twenty-something years on. Nat. Protoc. 1, 581–585. https://doi.org/10.1038/nprot.2006.83

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159. https://doi.org/10.1016/0003-2697(87)90021-2

Coffey, A.A., Fang, J., Grigson, P.S., 2018. Heroin self-administration as a function of time of day in rats. Psychopharmacology (Berl). 235, 3005–3015. https://doi.org/10.1007/s00213-018-4990-9

Conrad, K.L., Ford, K., Marinelli, M., Wolf, M.E., 2010. Dopamine receptor expression and distribution dynamically change in the rat nucleus accumbens after withdrawal from cocaine self-administration. Neuroscience 169, 182–94. https://doi.org/10.1016/j.neuroscience.2010.04.056

Conrad, K.L., Tseng, K.Y., Uejima, J.L., Reimers, J.M., Heng, L.J., Shaham, Y., Marinelli, M., Wolf, M.E., 2008. Formation of accumbens GluR2-lacking AMPA receptors mediates incubation of cocaine craving. Nature. https://doi.org/10.1038/nature06995

Counotte, D.S., Schiefer, C., Shaham, Y., O'Donnell, P., 2014. Time-dependent decreases in nucleus accumbens AMPA/NMDA ratio and incubation of sucrose craving in adolescent and adult rats. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-013-3294-3

Courtney, K.E., Schacht, J.P., Hutchison, K., Roche, D.J.O., Ray, L.A., 2016. Neural substrates of cue reactivity: association with treatment outcomes and relapse. Addict. Biol. 21, 3–22. https://doi.org/10.1111/adb.12314

D'Cunha, T.M., Daoud, E., Rizzo, D., Bishop, A.B., Russo, M., Mourra, G., Hamel, L., Sedki, F., Shalev, U., 2017. Augmentation of Heroin Seeking Following Chronic Food Restriction in the Rat: Differential Role for Dopamine Transmission in the Nucleus Accumbens Shell and Core. Neuropsychopharmacology. https://doi.org/10.1038/npp.2016.250

D'Cunha, T.M., Sedki, F., MacRi, J., Casola, C., Shalev, U., 2013. The effects of chronic food restriction on cue-induced heroin seeking in abstinent male rats. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-012-2810-1

de Araújo Costa Folha, O.A., Bahia, C.P., de Aguiar, G.P.S., Herculano, A.M., Coelho, N.L.G., de Sousa, M.B.C., Shiramizu, V.K.M., de Menezes Galvão, A.C., de Carvalho, W.A., Pereira, A., 2017. Effect of chronic stress during adolescence in prefrontal cortex structure and function. Behav. Brain Res. 326, 44–

51. https://doi.org/10.1016/j.bbr.2017.02.033

De Boer, J.Z., Hale, J.R., 2000. The geological origins of the oracle at Delphi, Greece. Geol. Soc. London, Spec. Publ. 171, 399–412. https://doi.org/10.1144/GSL.SP.2000.171.01.29

De Luca, M.A., Solinas, M., Bimpisidis, Z., Goldberg, S.R., Di Chiara, G., 2012. Cannabinoid facilitation of behavioral and biochemical hedonic taste responses. Neuropharmacology 63, 161–168. https://doi.org/10.1016/j.neuropharm.2011.10.018

de Rover, M., Brown, S.B.R.E., Boot, N., Hajcak, G., van Noorden, M.S., van der Wee, N.J.A., Nieuwenhuis, S., 2012. Beta receptor-mediated modulation of the late positive potential in humans. Psychopharmacology (Berl). 219, 971–9. https://doi.org/10.1007/s00213-011-2426-x

Degasperi, A., Birtwistle, M.R., Volinsky, N., Rauch, J., Kolch, W., Kholodenko, B.N., 2014. Evaluating strategies to normalise biological replicates of western blot data. PLoS One 9. https://doi.org/10.1371/journal.pone.0087293

Diehl, G.W., Wachtel, J.M., Paine, T.A., 2013. Cue-induced conditioned activity does not incubate but is mediated by the basolateral amygdala. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2013.01.003

Dikshtein, Y., Barnea, R., Kronfeld, N., Lax, E., Roth-Deri, I., Friedman, A., Gispan, I., Elharrar, E., Levy, S., Ben-Tzion, M., Yadid, G., 2013. β-Endorphin via the delta opioid receptor is a major factor in the incubation of cocaine craving. Neuropsychopharmacology. https://doi.org/10.1038/npp.2013.155

Dingess, P.M., Darling, R.A., Derman, R.C., Wulff, S.S., Hunter, M.L., Ferrario, C.R., Brown, T.E., 2017. Structural and Functional Plasticity within the Nucleus Accumbens and Prefrontal Cortex Associated with Time- Dependent Increases in Food Cue Seeking Behavior. https://doi.org/10.1038/

Diven, K., 1937. Certain determinants in the conditioning of anxiety reactions. J. Psychol. Interdiscip. Appl. https://doi.org/10.1080/00223980.1937.9917499

Dong, Y., Taylor, J.R., Wolf, M.E., Shaham, Y., 2017. Circuit and Synaptic Plasticity Mechanisms of Drug Relapse. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.1821-17.2017

Everitt, B.J., Robbins, T.W., 2016. Drug Addiction: Updating Actions to Habits to Compulsions Ten Years On. Annu. Rev. Psychol. 67, 23–50. https://doi.org/10.1146/annurev-psych-122414-033457

Fanous, S., Goldart, E.M., Theberge, F.R.M., Bossert, J.M., Shaham, Y., Hope, B.T., 2012. Role of Orbitofrontal Cortex Neuronal Ensembles in the Expression of Incubation of Heroin Craving. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.1914-12.2012

Fanous, S., Guez-Barber, D.H., Goldart, E.M., Schrama, R., Theberge, F.R.M., Shaham, Y., Hope, B.T., 2013. Unique gene alterations are induced in FACS-purified Fos-positive neurons activated during cue-induced relapse to heroin seeking. J. Neurochem. https://doi.org/10.1111/jnc.12074

Ferrario, C.R., Goussakov, I., Stutzmann, G.E., Wolf, M.E., 2012. Withdrawal from Cocaine Self-Administration Alters NMDA Receptor-Mediated Ca2+ Entry in Nucleus Accumbens Dendritic Spines. PLoS One 7, e40898. https://doi.org/10.1371/journal.pone.0040898

Ferrario, C.R., Loweth, J.A., Milovanovic, M., Ford, K.A., Galiñanes, G.L., Heng, L.J., Tseng, K.Y., Wolf, M.E., 2011. Alterations in AMPA receptor subunits and TARPs in the rat nucleus accumbens related to the formation of Ca 2+-permeable AMPA receptors during the incubation of cocaine craving. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2011.01.021

Field, A., 2013. Andy Field - Discovering Statistics Using SPSS. Lavoisier.Fr. https://doi.org/10.1111/j.1365-2648.2007.04270_1.x

Fischer-Smith, K.D., Houston, A.C.W., Rebec, G. V., 2012. Differential effects of cocaine access and withdrawal on glutamate type 1 transporter expression in rat nucleus accumbens core and shell. Neuroscience. https://doi.org/10.1016/j.neuroscience.2012.02.049

Fox, H.C., Talih, M., Malison, R., Anderson, G.M., Kreek, M.J., Sinha, R., 2005. Frequency of recent cocaine and alcohol use affects drug craving and associated responses to stress and drug-related cues. Psychoneuroendocrinology. https://doi.org/10.1016/j.psyneuen.2005.05.002

Freeman, W.M., Lull, M.E., Patel, K.M., Brucklacher, R.M., Morgan, D., Roberts, D.C.S., Vrana, K.E., 2010. Gene expression changes in the medial prefrontal cortex and nucleus accumbens following abstinence from cocaine self-administration. BMC Neurosci. https://doi.org/10.1186/1471-2202-11-29

Freeman, W.M., Patel, K.M., Brucklacher, R.M., Lull, M.E., Erwin, M., Morgan, D., Roberts, D.C.S., Vrana, K.E., 2008. Persistent alterations in mesolimbic gene expression with abstinence from cocaine self-administration. Neuropsychopharmacology. https://doi.org/10.1038/sj.npp.1301577

Funk, D., Coen, K., Tamadon, S., Hope, B.T., Shaham, Y., Lê, A.D., 2016. Role of Central Amygdala Neuronal Ensembles in Incubation of Nicotine Craving. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.1505-16.2016

Gancarz-Kausch, A.M., Adank, D.N., Dietz, D.M., 2014. Prolonged withdrawal following cocaine self-administration increases resistance to punishment in a cocaine binge. Sci. Rep. https://doi.org/10.1038/srep06876

Ghasemzadeh, M.B., Vasudevan, P., Giles, C., Purgianto, A., Seubert, C., Mantsch, J.R., 2011. Glutamatergic plasticity in medial prefrontal cortex and ventral tegmental area following extended-access cocaine self-administration. Brain Res. https://doi.org/10.1016/j.brainres.2011.06.041

Glueck, E., Ginder, D., Hyde, J., North, K., Grimm, J.W., 2017. Effects of dopamine D1 and D2 receptor agonists on environmental enrichment attenuated sucrose cue reactivity in rats.

Psychopharmacology (Berl). https://doi.org/10.1007/s00213-016-4516-2

Glynn, R.M., Rosenkranz, J.A., Wolf, M.E., Caccamise, A., Shroff, F., Smith, A.B., Loweth, J.A., 2018. Repeated restraint stress exposure during early withdrawal accelerates incubation of cue-induced cocaine craving. Addict. Biol. https://doi.org/10.1111/adb.12475

Gobin, C., Schwendt, M., 2017. The Effects of Extended-Access Cocaine Self-Administration on Working Memory Performance, Reversal Learning and Incubation of Cocaine-Seeking in Adult Male Rats HHS Public Access 5.

Goldfarb, E. V., Sinha, R., 2018. Drug-Induced Glucocorticoids and Memory for Substance Use. Trends Neurosci. 41, 853–868. https://doi.org/10.1016/j.tins.2018.08.005

Gould, A.T., Sacramento, A.D., Wroten, M.G., Miller, B.W., Von Jonquieres, G., Klugmann, M., Ben-Shahar, O., Szumlinski, K.K., 2015. Cocaine-elicited imbalances in ventromedial prefrontal cortex Homer1 versus Homer2 expression: Implications for relapse. Addict. Biol. https://doi.org/10.1111/adb.12088

Gozzi, A., Tessari, M., Dacome, L., Agosta, F., Lepore, S., Lanzoni, A., Cristofori, P., Pich, E.M., Corsi, M., Bifone, A., 2011. Neuroimaging evidence of altered fronto-cortical and striatal function after prolonged cocaine self-administration in the rat. Neuropsychopharmacology. https://doi.org/10.1038/npp.2011.129

Gray, J.M., Wilson, C.D., Lee, T.T.Y., Pittman, Q.J., Deussing, J.M., Hillard, C.J., McEwen, B.S., Schulkin, J., Karatsoreos, I.N., Patel, S., Hill, M.N., 2016. Sustained glucocorticoid exposure recruits cortico-limbic CRH signaling to modulate endocannabinoid function. Psychoneuroendocrinology. https://doi.org/10.1016/j.psyneuen.2016.01.004

Grimm, J.W., Barnes, J.L., Koerber, J., Glueck, E., Ginder, D., Hyde, J., Eaton, L., 2016. Effects of acute or chronic environmental enrichment on regional Fos protein expression following sucrose cue-reactivity testing in rats. Brain Struct. Funct. https://doi.org/10.1007/s00429-015-1074-z

Grimm, J.W., Buse, C., Manaois, M., Osincup, D., Fyall, A., Wells, B., 2006. Time-dependent dissociation of cocaine dose-response effects on sucrose craving and locomotion. Behav. Pharmacol. 17, 143–149. https://doi.org/10.1097/01.fbp.0000190686.23103.f8

Grimm, J.W., Fyall, A.M., Osincup, D.P., 2005. Incubation of sucrose craving: Effects of reduced training and sucrose pre-loading. Physiol. Behav. https://doi.org/10.1016/j.physbeh.2004.10.011

Grimm, J.W., Harkness, J.H., Ratliff, C., Barnes, J., North, K., Collins, S., 2011. Effects of systemic or nucleus accumbens-directed dopamine D1 receptor antagonism on sucrose seeking in rats. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-011-2210-y

Grimm, J.W., Hope, B.T., Wise, R.A., Shaham, Y., 2001. Incubation of cocaine craving after withdrawal. Nature 412, 141–142. https://doi.org/10.1038/35084134

Grimm, J.W., Lu, L., Hayashi, T., Hope, B.T., Su, T.-P., Shaham, Y., 2003. Time-dependent increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: implications for incubation of cocaine craving. J. Neurosci. 23, 742–7.

Grimm, J.W., Osincup, D., Wells, B., Manaois, M., Fyall, A., Buse, C., Harkness, J.H., 2008. Environmental enrichment attenuates cue-induced reinstatement of sucrose seeking in rats. Behav. Pharmacol. https://doi.org/10.1097/FBP.0b013e32831c3b18

Grimm, J.W., Shaham, Y., Hope, B.T., Grimm, J.W., 2002. Effect of cocaine and sucrose withdrawal period on extinction behavior, cue-induced reinstatement, and protein levels of the dopamine transporter and tyrosine hydroxylase in limbic and cortical areas in rats. Behav Pharmacol 13, 379–388.

Grimm, J.W., Weber, R., Barnes, J., Koerber, J., Dorsey, K., Glueck, E., 2013. Brief Exposure to Novel or Enriched Environments Reduces Sucrose Cue-Reactivity and Consumption in Rats after 1 or 30 Days of Forced Abstinence from Self-Administration. PLoS One. https://doi.org/10.1371/journal.pone.0054164

Gu, X., 2018. Incubation of craving: a Bayesian account. Neuropsychopharmacology 1. https://doi.org/10.1038/s41386-018-0108-7

Gueye, A.B., Allain, F., Samaha, A.-N., 2018. Intermittent intake of rapid cocaine injections promotes the risk of relapse and increases mesocorticolimbic BDNF levels during abstinence. Neuropsychopharmacology 1. https://doi.org/10.1038/s41386-018-0249-8

Guillem, K., Ahmed, S.H., 2018. Incubation of accumbal neuronal reactivity to cocaine cues during abstinence predicts individual vulnerability to relapse. Neuropsychopharmacology. https://doi.org/10.1038/npp.2017.224

Hagen, E.H., Sullivan, R.J., Schmidt, R., Morris, G., Kempter, R., Hammerstein, P., 2009. Ecology and neurobiology of toxin avoidance and the paradox of drug reward. Neuroscience 160, 69–84. https://doi.org/10.1016/j.neuroscience.2009.01.077

Halbout, B., Bernardi, R.E., Hansson, A.C., Spanagel, R., 2014. Incubation of Cocaine Seeking following Brief Cocaine Experience in Mice Is Enhanced by mGluR1 Blockade. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.1076-13.2014

Hamed, A., Boguszewski, P.M., 2018. Effects of Morphine and Other Opioid Ligands on Emission of Ultrasonic Vocalizations in Rats, in: Handbook of Behavioral Neuroscience. https://doi.org/10.1016/B978-0-12-809600-0.00031-7

Hamed, A., Taracha, E., Szyndler, J., Krzaścik, P., Lehner, M., Maciejak, P., Skórzewska, A., Płaźnik, A., 2012. The effects of morphine and morphine conditioned context on 50kHz ultrasonic vocalisation in rats. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2012.01.053

Häring, M., Guggenhuber, S., Lutz, B., 2012. Neuronal populations mediating the effects of endocannabinoids on stress and emotionality. Neuroscience 204, 145–158. https://doi.org/10.1016/j.neuroscience.2011.12.035

Harkness, J.H., Webb, S., Grimm, J.W., 2010. Abstinence-dependent transfer of lithium chloride-induced sucrose aversion to a sucrose-paired cue in rats. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-009-1755-5

Harkness, J.H., Wells, J., Webb, S., Grimm, J.W., 2016. Extended exposure to environmental cues, but not to sucrose, reduces sucrose cue reactivity in rats. Learn. Behav. https://doi.org/10.3758/s13420-015-0190-1

Higuera-Matas, A., Luisa Soto-Montenegro, M., Del Olmo, N., Miguéns, M., Torres, I., José Vaquero, J., Sánchez, J., García-Lecumberri, C., Desco, M., Ambrosio, E., 2008. Augmented acquisition of cocaine self-administration and altered brain glucose metabolism in adult female but not male rats exposed to a cannabinoid agonist during adolescence. Neuropsychopharmacology 33. https://doi.org/10.1038/sj.npp.1301467

Hill, M.N., Tasker, J.G., 2011. Endocannabinoid signaling, glucocorticoid-mediated negative feedback, and regulation of the hypothalamic-pituitary-adrenal axis. Neuroscience. https://doi.org/S0306-4522(11)01427-8 [pii]\r10.1016/j.neuroscience.2011.12.030

Hollander, J.A., Carelli, R.M., 2007. Cocaine-Associated Stimuli Increase Cocaine Seeking and Activate Accumbens Core Neurons after Abstinence. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.3667-06.2007

Hollander, J.A., Carelli, R.M., 2005. Abstinence from cocaine self-administration heightens neural encoding of goal-directed behaviors in the accumbens. Neuropsychopharmacology. https://doi.org/10.1038/sj.npp.1300748

Hunt, W.A., Barnett, L.W., Branch, L.G., 1971. Relapse rates in addiction programs. J. Clin. Psychol. https://doi.org/10.1002/1097-4679(197110)27:4<455::AID-JCLP2270270412>3.0.CO;2-R

Izquierdo, A., Brigman, J.L., Radke, A.K., Rudebeck, P.H., Holmes, A., 2017. The neural basis of reversal learning: An updated perspective. Neuroscience 345, 12–26. https://doi.org/10.1016/j.neuroscience.2016.03.021

James, M.H., Stopper, C.M., Zimmer, B.A., Koll, N.E., Bowrey, H.E., Aston-Jones, G., 2018. Increased Number and Activity of a Lateral Subpopulation of Hypothalamic Orexin/Hypocretin Neurons Underlies the Expression of an Addicted State in Rats. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2018.07.022

Jones, J.L., Wheeler, R.A., Carelli, R.M., 2008. Behavioral responding and nucleus accumbens cell firing are unaltered following periods of abstinence from sucrose. Synapse. https://doi.org/10.1002/syn.20486

Kahathuduwa, C.N., Davis, T., O'Boyle, M., Boyd, L.A., Chin, S.H., Paniukov, D., Binks, M., 2018. Effects of 3-week total meal replacement vs. typical food-based diet on human brain functional magnetic resonance imaging food-cue reactivity and functional connectivity in people with obesity. Appetite. https://doi.org/10.1016/j.appet.2017.09.025

Karlsson, R.M., Kircher, D.M., Shaham, Y., O'Donnell, P., 2013. Exaggerated cue-induced reinstatement of cocaine seeking but not incubation of cocaine craving in a developmental rat model of schizophrenia. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-012-2882-y

Kasanetz, F., Deroche-Gamonet, V., Berson, N., Balado, E., Lafourcade, M., Manzoni, O., Vincenzo Piazza, P., 2010. Transition to addiction is associated with a persistent impairment in synaptic plasticity. Science (80-.). https://doi.org/10.1126/science.1187801

Keith, D., 2008. Excitation control: balancing PSD-95 function at the synapse. Front. Mol. Neurosci. 1. https://doi.org/10.3389/neuro.02.004.2008

Keller, J., Young, C.B., Kelley, E., Prater, K., Levitin, D.J., Menon, V., 2013. Trait anhedonia is associated with reduced reactivity and connectivity of mesolimbic and paralimbic reward pathways. J. Psychiatr. Res. 47, 1319–1328. https://doi.org/10.1016/j.jpsychires.2013.05.015

Kerstetter, K.A., Aguilar, V.R., Parrish, A.B., Kippin, T.E., 2008. Protracted time-dependent increases in cocaine-seeking behavior during cocaine withdrawal in female relative to male rats. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-008-1089-8

Kinnavane, L., Amin, E., Horne, M., Aggleton, J.P., 2014. Mapping parahippocampal systems for recognition and recency memory in the absence of the rat hippocampus 40, 3720–3734. https://doi.org/10.1111/ejn.12740

Kirschmann, E.K., Pollock, M.W., Nagarajan, V., Torregrossa, M.M., 2017. Effects of Adolescent Cannabinoid Self-Administration in Rats on Addiction-Related Behaviors and Working Memory. Neuropsychopharmacology. https://doi.org/10.1038/npp.2016.178

Koya, E., Uejima, J.L., Wihbey, K.A., Bossert, J.M., Hope, B.T., Shaham, Y., 2009. Role of ventral medial prefrontal cortex in incubation of cocaine craving. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2008.04.022

Krasnova, I.N., Gerra, M.C., Walther, D., Jayanthi, S., Ladenheim, B., McCoy, M.T., Brannock, C., Cadet, J.L., 2017. Compulsive methamphetamine taking in the presence of punishment is associated with increased oxytocin expression in the nucleus accumbens of rats. Sci. Rep. https://doi.org/10.1038/s41598-017-08898-8

Krasnova, I.N., Marchant, N.J., Ladenheim, B., McCoy, M.T., Panlilio, L. V., Bossert, J.M., Shaham, Y., Cadet, J.L., 2014. Incubation of methamphetamine and palatable food craving after punishment-induced abstinence. Neuropsychopharmacology. https://doi.org/10.1038/npp.2014.50

Kvetnansky, R., Sabban, E.L., Palkovits, M., 2009. Catecholaminergic systems in stress: structural and molecular genetic approaches. Physiol. Rev. 89, 535–606. https://doi.org/10.1152/physrev.00042.2006

Ladner, C.L., Yang, J., Turner, R.J., Edwards, R.A., 2004. Visible fluorescent detection of proteins in polyacrylamide gels without staining. Anal. Biochem. 326, 13–20. https://doi.org/10.1016/j.ab.2003.10.047

Lee, B.R., Ma, Y.Y., Huang, Y.H., Wang, X., Otaka, M., Ishikawa, M., Neumann, P.A., Graziane, N.M., Brown, T.E., Suska, A., Guo, C., Lobo, M.K., Sesack, S.R., Wolf, M.E., Nestler, E.J., Shaham, Y., Schlüter, O.M., Dong, Y., 2013. Maturation of silent synapses in amygdala-accumbens projection contributes to incubation of cocaine craving. Nat. Neurosci. https://doi.org/10.1038/nn.3533

Leventhal, A.M., Brightman, M., Ameringer, K.J., Greenberg, J., Mickens, L., Ray, L.A., Sun, P., Sussman, S., 2010. Anhedonia associated with stimulant use and dependence in a population-based sample of American adults. Exp. Clin. Psychopharmacol. 18, 562–569. https://doi.org/10.1037/a0021964

Li, C., Frantz, K.J., 2009. Attenuated incubation of cocaine seeking in male rats trained to self-administer cocaine during periadolescence. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-009-1502-y

Li, P., Wu, P., Xin, X., Fan, Y.L., Wang, G. Bin, Wang, F., Ma, M.Y., Xue, M.M., Luo, Y.X., Yang, F. De, Bao, Y.P., Shi, J., Sun, H.Q., Lu, L., 2015c. Incubation of alcohol craving during abstinence in patients with alcohol dependence. Addict. Biol. https://doi.org/10.1111/adb.12140

Li, Q., Wang, Y., Zhang, Y., Li, W., Zhu, J., Zheng, Y., Chen, J., Zhao, L., Zhou, Z., Liu, Y., Wang, W., Tian, J., 2013a. Assessing Cue-Induced Brain Response as a Function of Abstinence Duration in Heroin-Dependent Individuals: An Event-Related fMRI Study. PLoS One 8. https://doi.org/10.1371/journal.pone.0062911

Li, X., Caprioli, D., Marchant, N.J., 2015a. Recent updates on incubation of drug craving: A mini-review. Addict. Biol. https://doi.org/10.1111/adb.12205

Li, X., DeJoseph, M.R., Urban, J.H., Bahi, A., Dreyer, J.-L., Meredith, G.E., Ford, K.A., Ferrario, C.R., Loweth, J.A., Wolf, M.E., 2013b. Different Roles of BDNF in Nucleus Accumbens Core versus Shell during the Incubation of Cue-Induced Cocaine Craving and Its Long-Term Maintenance. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.3082-12.2013

Li, X., Rubio, F.J., Zeric, T., Bossert, J.M., Kambhampati, S., Cates, H.M., Kennedy, P.J., Liu, Q.-R., Cimbro, R., Hope, B.T., Nestler, E.J., Shaham, Y., 2015d. Incubation of Methamphetamine Craving Is Associated with Selective Increases in Expression of Bdnf and Trkb, Glutamate Receptors, and Epigenetic Enzymes in Cue-Activated Fos-Expressing Dorsal Striatal Neurons. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.1022-15.2015

Li, X., Venniro, M., Shaham, Y., 2016. Translational Research on Incubation of Cocaine Craving. JAMA Psychiatry. https://doi.org/10.1001/jamapsychiatry.2016.2110

Li, X., Witonsky, K., Lofaro, O.M., Surjono, F., Zhang, J., Bossert, J.M., Shaham, Y., 2018. Role of anterior intralaminar nuclei of thalamus projections to dorsomedial striatum in incubation of methamphetamine craving. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.2873-17.2018

Li, X., Wolf, M.E., 2015. Multiple faces of BDNF in cocaine addiction. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2014.11.018

Li, X., Zeric, T., Kambhampati, S., Bossert, J.M., Shaham, Y., 2015b. The Central Amygdala Nucleus is Critical for Incubation of Methamphetamine Craving. Neuropsychopharmacology. https://doi.org/10.1038/npp.2014.320

Li, Y.-Q., Li, F.-Q., Wang, X.-Y., Wu, P., Zhao, M., Xu, C.-M., Shaham, Y., Lu, L., 2008. Central Amygdala Extracellular Signal-Regulated Kinase Signaling Pathway Is Critical to Incubation of Opiate Craving. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.3027-08.2008

Lorenzo, M.P., Navarrete, A., Balderas, C., Garcia, A., 2013. Optimization and validation of a CE-LIF method for amino acid determination in biological samples. J. Pharm. Biomed. Anal. 73, 116–124. https://doi.org/10.1016/j.jpba.2012.03.017

Lorenzo, M.P., Villaseñor, A., Ramamoorthy, A., Garcia, A., 2013. Optimization and validation of a capillary electrophoresis laser-induced fluorescence method for amino acids determination in human plasma: Application to bipolar disorder study. Electrophoresis 34, 1701–1709. https://doi.org/10.1002/elps.201200632

Loweth, J.A., Scheyer, A.F., Milovanovic, M., Lacrosse, A.L., Flores-Barrera, E., Werner, C.T., Li, X., Ford, K.A., Le, T., Olive, M.F., Szumlinski, K.K., Tseng, K.Y., Wolf, M.E., 2014a. Synaptic depression via mGluR1 positive allosteric modulation suppresses cue-induced cocaine craving. Nat. Neurosci. https://doi.org/10.1038/nn.3590

Loweth, J.A., Tseng, K.Y., Wolf, M.E., 2014b. Adaptations in AMPA receptor transmission in the nucleus accumbens contributing to incubation of cocaine craving. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2013.04.061

Lu, L., Dempsey, J., Shaham, Y., Hope, B.T., 2005a. Differential long-term neuroadaptations of glutamate receptors in the basolateral and central amygdala after withdrawal from cocaine self-administration in rats. J. Neurochem. https://doi.org/10.1111/j.1471-4159.2005.03178.x

Lu, L., Grimm, J.W., Hope, B.T., Shaham, Y., 2004. Incubation of cocaine craving after withdrawal: A review of preclinical data. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2004.06.027

Lu, L., Grimm, J.W., Shaham, Y., Hope, B.T., 2003. Molecular neuroadaptations in the accumbens and

ventral tegmental area during the first 90 days of forced abstinence from cocaine self-administration in rats. J. Neurochem. https://doi.org/10.1046/j.1471-4159.2003.01824.x

Lu, L., Hope, B.T., Dempsey, J., Liu, S.Y., Bessert, J.M., Shaham, Y., 2005b. Central amygdala ERK signaling pathway is critical to incubation of cocaine craving. Nat. Neurosci. https://doi.org/10.1038/nn1383

Lu, L., Koya, E., Zhai, H., Hope, B.T., Shaham, Y., 2006. Role of ERK in cocaine addiction. Trends Neurosci. https://doi.org/10.1016/j.tins.2006.10.005

Lu, L., Uejima, J.L., Gray, S.M., Bossert, J.M., Shaham, Y., 2007. Systemic and Central Amygdala Injections of the mGluR2/3 Agonist LY379268 Attenuate the Expression of Incubation of Cocaine Craving. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2006.04.011

Lu, L., Wang, X., Wu, P., Xu, C., Zhao, M., Morales, M., Harvey, B.K., Hoffer, B.J., Shaham, Y., 2009. Role of ventral tegmental area glial cell line-derived neurotrophic factor in incubation of cocaine craving. Biol. Psychiatry 66, 137–45. https://doi.org/10.1016/j.biopsych.2009.02.009

Lubbers, B.R., Matos, M.R., Horn, A., Visser, E., Van der Loo, R.C., Gouwenberg, Y., Meerhoff, G.F., Frischknecht, R., Seidenbecher, C.I., Smit, A.B., Spijker, S., van den Oever, M.C., 2016. The Extracellular Matrix Protein Brevican Limits Time-Dependent Enhancement of Cocaine Conditioned Place Preference. Neuropsychopharmacology 41, 1907–16. https://doi.org/10.1038/npp.2015.361

Luís, C., Cannella, N., Spanagel, R., Köhr, G., 2017. Persistent strengthening of the prefrontal cortex – nucleus accumbens pathway during incubation of cocaine-seeking behavior. Neurobiol. Learn. Mem. https://doi.org/10.1016/j.nlm.2016.10.003

Lull, M.E., Erwin, M.S., Morgan, D., Roberts, D.C.S., Vrana, K.E., Freeman, W.M., 2009. Persistent proteomic alterations in the medial prefrontal cortex with abstinence from cocaine self-administration. Proteomics. Clin. Appl. 3, 462–472. https://doi.org/10.1002/prca.200800055

Ma, Y.-Y., Wang, X., Huang, Y., Marie, H., Nestler, E.J., Schlüter, O.M., Dong, Y., 2016. Re-silencing of silent synapses unmasks anti-relapse effects of environmental enrichment. Proc. Natl. Acad. Sci. 113, 5089–5094. https://doi.org/10.1073/pnas.1524739113

Ma, Y.Y., Lee, B.R., Wang, X., Guo, C., Liu, L., Cui, R., Lan, Y., Balcita-Pedicino, J.J., Wolf, M.E., Sesack, S.R., Shaham, Y., Schlüter, O.M., Huang, Y.H., Dong, Y., 2014. Bidirectional modulation of incubation of cocaine craving by silent synapse-based remodeling of prefrontal cortex to accumbens projections. Neuron. https://doi.org/10.1016/j.neuron.2014.08.023

Madsen, H.B., Zbukvic, I.C., Luikinga, S.J., Lawrence, A.J., Kim, J.H., 2017. Extinction of conditioned cues attenuates incubation of cocaine craving in adolescent and adult rats. Neurobiol. Learn. Mem. https://doi.org/10.1016/j.nlm.2016.09.002

Mahler, S. V, Smith, K.S., Berridge, K.C., 2007. Endocannabinoid Hedonic Hotspot for Sensory Pleasure: Anandamide in Nucleus Accumbens Shell Enhances 'Liking' of a Sweet Reward. Neuropsychopharmacology 32, 2267–2278. https://doi.org/10.1038/sj.npp.1301376

Mameli, M., Halbout, B., Creton, C., Engblom, D., Parkitna, J.R., Spanagel, R., Lüscher, C., 2009. Cocaine-evoked synaptic plasticity: Persistence in the VTA triggers adaptations in the NAc. Nat. Neurosci. https://doi.org/10.1038/nn.2367

Mantsch, J.R., Vranjkovic, O., Twining, R.C., Gasser, P.J., McReynolds, J.R., Blacktop, J.M., 2014. Neurobiological mechanisms that contribute to stress-related cocaine use. Neuropharmacology 76, 383–394. https://doi.org/10.1016/j.neuropharm.2013.07.021

Mantsch, J.R., Yuferov, V., Mathieu-Kia, A.M., Ho, A., Kreek, M.J., 2003. Neuroendocrine alterations in a high-dose, extended-access rat self-administration model of escalating cocaine use. Psychoneuroendocrinology. https://doi.org/10.1016/S0306-4530(02)00088-4

Marchant, N.J., Li, X., Shaham, Y., 2013. Recent developments in animal models of drug relapse. Curr. Opin. Neurobiol. https://doi.org/10.1016/j.conb.2013.01.003

Markou, A., Li, J., Tse, K., Li, X., 2018. Cue-induced nicotine-seeking behavior after withdrawal with or without extinction in rats. Addict. Biol. https://doi.org/10.1111/adb.12480

Martin, C.K., O'Neil, P.M., Pawlow, L., 2006. Changes in food cravings during low-calorie and very-low-calorie diets. Obesity. https://doi.org/10.1038/oby.2006.14

Massart, R., Barnea, R., Dikshtein, Y., Suderman, M., Meir, O., Hallett, M., Kennedy, P., Nestler, E.J., Szyf, M., Yadid, G., 2015. Role of DNA Methylation in the Nucleus Accumbens in Incubation of Cocaine Craving. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.3053-14.2015

Massey, A., Hill, A.J., 2012. Dieting and food craving. A descriptive, quasi-prospective study. Appetite. https://doi.org/10.1016/j.appet.2012.01.020

McCue, D.L., Kasper, J.M., Ara, A., Hommel, J.D., 2018. Incubation of feeding behavior is regulated by neuromedin U receptor 2 in the paraventricular nucleus of the hypothalamus. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2018.08.015

McCutcheon, J.E., Wang, X., Tseng, K.Y., Wolf, M.E., Marinelli, M., 2011. Calcium-Permeable AMPA Receptors Are Present in Nucleus Accumbens Synapses after Prolonged Withdrawal from Cocaine Self-Administration But Not Experimenter-Administered Cocaine. J. Neurosci. 31, 5737–5743. https://doi.org/10.1523/JNEUROSCI.0350-11.2011

McLaughlin, R.J., Gobbi, G., 2012. Cannabinoids and emotionality: a neuroanatomical perspective. Neuroscience 204, 134–144. https://doi.org/10.1016/j.neuroscience.2011.07.052

Mijakowska, Z., Łukasiewicz, K., Ziółkowska, M., Lipiński, M., Trąbczyńska, A., Matuszek, Ż., Łęski, S., Radwanska, K., 2017. Autophosphorylation of alpha isoform of calcium/calmodulin-dependent

kinase II regulates alcohol addiction-related behaviors. Addict. Biol. https://doi.org/10.1111/adb.12327

Miller, B.W., Wroten, M.G., Sacramento, A.D., Silva, H.E., Shin, C.B., Vieira, P.A., Ben-Shahar, O., Kippin, T.E., Szumlinski, K.K., 2017. Cocaine craving during protracted withdrawal requires PKCε priming within vmPFC. Addict. Biol. https://doi.org/10.1111/adb.12354

Morena, M., Leitl, K.D., Vecchiarelli, H.A., Gray, J.M., Campolongo, P., Hill, M.N., 2016a. Emotional arousal state influences the ability of amygdalar endocannabinoid signaling to modulate anxiety. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2016.08.020

Morena, M., Patel, S., Bains, J.S., Hill, M.N., 2016b. Neurobiological Interactions Between Stress and the Endocannabinoid System. Neuropsychopharmacology 41, 80–102. https://doi.org/10.1038/npp.2015.166

Müller Ewald, V.A., De Corte, B.J., Gupta, S.C., Lillis, K. V., Narayanan, N.S., Wemmie, J.A., LaLumiere, R.T., 2018. Attenuation of cocaine seeking in rats via enhancement of infralimbic cortical activity using stable step-function opsins. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-018-4964-y

Murray, J.E., Belin-Rauscent, A., Simon, M., Giuliano, C., Benoit-Marand, M., Everitt, B.J., Belin, D., 2015. Basolateral and central amygdala differentially recruit and maintain dorsolateral striatum-dependent cocaine-seeking habits. Nat. Commun. 6, 10088. https://doi.org/10.1038/ncomms10088

Nagelhout, G.E., Hummel, K., de Goeij, M.C.M., de Vries, H., Kaner, E., Lemmens, P., 2017. How economic recessions and unemployment affect illegal drug use: A systematic realist literature review. Int. J. Drug Policy. https://doi.org/10.1016/j.drugpo.2017.03.013

Nava, F., Caldiroli, E., Premi, S., Lucchini, A., 2006. Relationship between plasma cortisol levels, withdrawal symptoms and craving in abstinent and treated heroin addicts. J Addict Dis. https://doi.org/10.1300/J069v25n02_02

Neumaier, J.F., McDevitt, R.A., Polis, I.Y., Parsons, L.H., 2009. Acquisition of and withdrawal from cocaine self-administration regulates 5-HT $_{1B}$ mRNA expression in rat striatum. J. Neurochem. 111, 217–227. https://doi.org/10.1111/j.1471-4159.2009.06313.x

Nugent, A.L., Anderson, E.M., Larson, E.B., Self, D.W., 2017. Incubation of cue-induced reinstatement of cocaine, but not sucrose, seeking in C57BL/6J mice. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2017.06.017

Nutt, D., King, L.A., Saulsbury, W., Blakemore, C., 2007. Development of a rational scale to assess the harm of drugs of potential misuse. Lancet 369, 1047–1053. https://doi.org/10.1016/S0140-6736(07)60464-4

Obesity: We need to move beyond sugar, 2016. . Lancet 387, 199. https://doi.org/10.1016/S0140-6736(16)00091-X

Orio, L., Edwards, S., George, O., Parsons, L.H., Koob, G.F., 2009. A role for the endocannabinoid system in the increased motivation for cocaine in extended-access conditions. J. Neurosci. 29, 4846–57. https://doi.org/10.1523/JNEUROSCI.0563-09.2009

Pacchioni, A.M., Gabriele, A., See, R.E., 2011. Dorsal striatum mediation of cocaine-seeking after withdrawal from short or long daily access cocaine self-administration in rats. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2010.12.014

Pape, H.C., Pare, D., 2010. Plastic Synaptic Networks of the Amygdala for the Acquisition, Expression, and Extinction of Conditioned Fear. Physiol. Rev. 90, 419–463. https://doi.org/10.1152/physrev.00037.2009

Parvaz, M.A., Moeller, S.J., Goldstein, R.Z., 2016. Incubation of cue-induced craving in adults addicted to cocaine measured by electroencephalography. JAMA Psychiatry. https://doi.org/10.1001/jamapsychiatry.2016.2181

Paxinos, G., Watson, C., 2007. The rat brain in stereotaxic coordinates, Academic Press.

Pentkowski, N.S., Duke, F.D., Weber, S.M., Pockros, L.A., Teer, A.P., Hamilton, E.C., Thiel, K.J., Neisewander, J.L., 2010. Stimulation of Medial Prefrontal Cortex Serotonin 2C (5-HT2C) Receptors Attenuates Cocaine-Seeking Behavior. Neuropsychopharmacology 35, 2037–2048. https://doi.org/10.1038/npp.2010.72

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids Res. 29, e45.

Piasecki, T.M., Niaura, R., Shadel, W.G., Abrams, D., Goldstein, M., Fiore, M.C., Baker, T.B., 2000. Smoking withdrawal dynamics in unaided quitters. J. Abnorm. Psychol. https://doi.org/10.1037/0021-843X.109.1.74

Pickens, C.L., Airavaara, M., Theberge, F., Fanous, S., Hope, B.T., Shaham, Y., 2011. Neurobiology of the incubation of drug craving. Trends Neurosci. https://doi.org/10.1016/j.tins.2011.06.001

Pickens, C.L., Golden, S.A., Adams-Deutsch, T., Nair, S.G., Shaham, Y., 2009. Long-Lasting Incubation of Conditioned Fear in Rats. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2008.12.010

Poirier, G.L., Amin, E., Aggleton, J.P., 2008. Qualitatively Different Hippocampal Subfield Engagement Emerges with Mastery of a Spatial Memory Task by Rats 28, 1034–1045. https://doi.org/10.1523/JNEUROSCI.4607-07.2008

Protzner, A.B., McIntosh, A.R., 2006. Testing effective connectivity changes with structural equation modeling: What does a bad model tell us? Hum. Brain Mapp. 27, 935–947. https://doi.org/10.1002/hbm.20233

Purgianto, A., Loweth, J.A., Miao, J.J., Milovanovic, M., Wolf, M.E., 2016. Surface expression of GABAAreceptors in the rat nucleus accumbens is increased in early but not late withdrawal from extended-access cocaine self-administration. Brain Res. https://doi.org/10.1016/j.brainres.2016.04.014

Purgianto, A., Weinfeld, M.E., Wolf, M.E., 2017. Prolonged withdrawal from cocaine self-administration affects prefrontal cortex- and basolateral amygdala–nucleus accumbens core circuits but not accumbens GABAergic local interneurons. Addict. Biol. https://doi.org/10.1111/adb.12430

Radwanska, K., Wrobel, E., Korkosz, A., Rogowski, A., Kostowski, W., Bienkowski, P., Kaczmarek, L., 2008. Alcohol relapse induced by discrete cues activates components of AP-1 transcription factor and ERK pathway in the rat basolateral and central amygdala. Neuropsychopharmacology. https://doi.org/10.1038/sj.npp.1301567

Ramikie, T.S., Patel, S., 2012. Endocannabinoid signaling in the amygdala: Anatomy, synaptic signaling, behavior, and adaptations to stress. Neuroscience. https://doi.org/10.1016/j.neuroscience.2011.08.037

Ren, Z.Y., Zhang, X.L., Liu, Y., Zhao, L.Y., Shi, J., Bao, Y., Zhang, X.Y., Kosten, T.R., Lu, L., 2009. Diurnal variation in cue-induced responses among protracted abstinent heroin users. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2008.08.023

Roozendaal, B., McEwen, B.S., Chattarji, S., 2009. Stress, memory and the amygdala. Nat. Rev. Neurosci. https://doi.org/10.1038/nrn2651

Roozendaal, B., McGaugh, J.L., 2011. Memory Modulation. Behav. Neurosci. https://doi.org/10.1037/a0026187

Ruijter, J.M., Ramakers, C., Hoogaars, W.M.H., Karlen, Y., Bakker, O., van den hoff, M.J.B., Moorman, A.F.M., 2009. Amplification efficiency: Linking baseline and bias in the analysis of quantitative PCR data. Nucleic Acids Res. 37. https://doi.org/10.1093/nar/gkp045

Scheyer, A.F., Loweth, J.A., Christian, D.T., Uejima, J., Rabei, R., Le, T., Dolubizno, H., Stefanik, M.T., Murray, C.H., Sakas, C., Wolf, M.E., 2016. AMPA Receptor Plasticity in Accumbens Core Contributes to Incubation of Methamphetamine Craving. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2016.04.003

Seamans, J.K., Lapish, C.C., Durstewitz, D., 2008. Comparing the prefrontal cortex of rats and primates: Insights from electrophysiology. Neurotox. Res. 14, 249–262. https://doi.org/10.1007/BF03033814

Serrano, A., Pavon, F.J., Buczynski, M.W., Schlosburg, J., Natividad, L.A., Polis, I.Y., Stouffer, D.G., Zorrilla, E.P., Roberto, M., Cravatt, B.F., Martin-Fardon, R., de Fonseca, F.R., Parsons, L.H., 2018. Deficient endocannabinoid signaling in the central amygdala contributes to alcohol dependence-related anxiety-like behavior and excessive alcohol intake. Neuropsychopharmacology 1. https://doi.org/10.1038/s41386-018-0055-3

Shalev, U., Morales, M., Hope, B., Yap, J., Shaham, Y., 2001. Time-dependent changes in extinction behavior and stress-induced reinstatement of drug seeking following withdrawal from heroin in rats. Psychopharmacology (Berl). https://doi.org/10.1007/s002130100748

Shepard, J.D., Bossert, J.M., Liu, S.Y., Shaham, Y., 2004. The anxiogenic drug yohimbine reinstates methamphetamine seeking in a rat model of drug relapse. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2004.02.032

Shin, C.B., Serchia, M.M., Shahin, J.R., Ruppert-Majer, M.A., Kippin, T.E., Szumlinski, K.K., 2016. Incubation of cocaine-craving relates to glutamate over-flow within ventromedial prefrontal cortex. Neuropharmacology. https://doi.org/10.1016/j.neuropharm.2015.10.038

Shinohara, Y., Inui, T., Yamamoto, T., Shimura, T., 2009. Cannabinoid in the nucleus accumbens enhances the intake of palatable solution. Neuroreport 20, 1382–1385. https://doi.org/10.1097/WNR.0b013e3283318010

Siller-Pérez, C., Serafín, N., Prado-Alcalá, R.A., Roozendaal, B., Quirarte, G.L., 2017. Glucocorticoid administration into the dorsolateral but not dorsomedial striatum accelerates the shift from a spatial toward procedural memory. Neurobiol. Learn. Mem. 141, 124–133. https://doi.org/10.1016/j.nlm.2017.03.020

Singer, B.F., Fadanelli, M., Kawa, A.B., Robinson, T.E., 2017. Are cocaine-seeking "habits" necessary for the development of addiction-like behavior in rats? J. Neurosci. 2458–17. https://doi.org/10.1523/JNEUROSCI.2458-17.2017

Sinha, R., 2011. New findings on biological factors predicting addiction relapse vulnerability. Curr. Psychiatry Rep. https://doi.org/10.1007/s11920-011-0224-0

Smith, E.S., Fabian, P., Rosenthal, A., Kaddour-Djebbar, A., Lee, H.J., 2015. The roles of central amygdala D1 and D2 receptors on attentional performance in a five-choice task. Behav. Neurosci. 129, 564–75. https://doi.org/10.1037/bne0000077

Smith, R.J., Aston-Jones, G., 2008. Noradrenergic transmission in the extended amygdala: Role in increased drug-seeking and relapse during protracted drug abstinence. Brain Struct. Funct. https://doi.org/10.1007/s00429-008-0191-3

Steiner, N., Rossetti, C., Sakurai, T., Yanagisawa, M., de Lecea, L., Magistretti, P.J., Halfon, O., Boutrel, B., 2018. Hypocretin/orexin deficiency decreases cocaine abuse liability. Neuropharmacology 133, 395–403. https://doi.org/10.1016/j.neuropharm.2018.02.010

Sun, A., Zhuang, D., Zhu, H., Lai, M., Chen, W., Liu, H., Zhang, F., Zhou, W., 2015. Decrease of phosphorylated CREB and ERK in nucleus accumbens is associated with the incubation of heroin seeking induced by cues after withdrawal. Neurosci. Lett.

https://doi.org/10.1016/j.neulet.2015.02.048

Sun, Y., Pan, Z., Ma, Y., 2017. Increased entrances to side compartments indicate incubation of craving in morphine-induced rat and tree shrew CPP models. Pharmacol. Biochem. Behav. https://doi.org/10.1016/j.pbb.2017.07.007

Swinford-Jackson, S.E., Anastasio, N.C., Fox, R.G., Stutz, S.J., Cunningham, K.A., 2016. Incubation of cocaine cue reactivity associates with neuroadaptations in the cortical serotonin (5-HT) 5-HT2Creceptor (5-HT2CR) system. Neuroscience. https://doi.org/10.1016/j.neuroscience.2016.02.052

Szumlinski, K.K., Ary, A.W., Shin, C.B., Wroten, M.G., Courson, J., Miller, B.W., Ruppert-Majer, M., Hiller, J.W., Shahin, J.R., Ben-Shahar, O., Kippin, T.E., 2018. PI3K activation within ventromedial prefrontal cortex regulates the expression of drug-seeking in two rodent species. Addict. Biol. https://doi.org/10.1111/adb.12696

Tang, D.W., Fellows, L.K., Small, D.M., Dagher, A., 2012. Food and drug cues activate similar brain regions: A meta-analysis of functional MRI studies. Physiol. Behav. 106, 317–324. https://doi.org/10.1016/j.physbeh.2012.03.009

Terrier, J., Lüscher, C., Pascoli, V., 2016. Cell-Type Specific Insertion of GluA2-Lacking AMPARs with Cocaine Exposure Leading to Sensitization, Cue-Induced Seeking, and Incubation of Craving. Neuropsychopharmacology. https://doi.org/10.1038/npp.2015.345

Theberge, F.R., Li, X., Kambhampati, S., Pickens, C.L., St. Laurent, R., Bossert, J.M., Baumann, M.H., Hutchinson, M.R., Rice, K.C., Watkins, L.R., Shaham, Y., 2013. Effect of chronic delivery of the toll-like receptor 4 antagonist (+)-naltrexone on incubation of heroin craving. Biol. Psychiatry. https://doi.org/10.1016/j.biopsych.2012.12.019

Theberge, F.R.M., Pickens, C.L., Goldart, E., Fanous, S., Hope, B.T., Liu, Q.R., Shaham, Y., 2012. Association of time-dependent changes in mu opioid receptor mRNA, but not BDNF, TrkB, or MeCP2 mRNA and protein expression in the rat nucleus accumbens with incubation of heroin craving. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-012-2784-z

Thiel, K.J., Painter, M.R., Pentkowski, N.S., Mitroi, D., Crawford, C.A., Neisewander, J.L., 2012. Environmental enrichment counters cocaine abstinence-induced stress and brain reactivity to cocaine cues but fails to prevent the incubation effect. Addict. Biol. https://doi.org/10.1111/j.1369-1600.2011.00358.x

Torres, O. V., Jayanthi, S., Ladenheim, B., McCoy, M.T., Krasnova, I.N., Cadet, J.L., 2017. Compulsive methamphetamine taking under punishment is associated with greater cue-induced drug seeking in rats. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2017.03.009

Uejima, J.L., Bossert, J.M., Poles, G.C., Lu, L., 2007. Systemic and central amygdala injections of the mGluR2/3agonist LY379268 attenuate the expression of incubation of sucrose craving in rats. Behav. Brain Res. https://doi.org/10.1016/j.bbr.2007.04.019

Ulrich-Lai, Y.M., Herman, J.P., 2009. Neural regulation of endocrine and autonomic stress responses. Nat. Rev. Neurosci. https://doi.org/10.1038/nrn2647

Valjent, E., Bertran-Gonzalez, J., Aubier, B., Greengard, P., Hervé, D., Girault, J.A., 2010. Mechanisms of locomotor sensitization to drugs of abuse in a two-injection protocol. Neuropsychopharmacology. https://doi.org/10.1038/npp.2009.143

Van Waes, V., Beverley, J.A., Siman, H., Tseng, K.Y., Steiner, H., 2012. CB1 Cannabinoid Receptor Expression in the Striatum: Association with Corticostriatal Circuits and Developmental Regulation. Front. Pharmacol. 3, 21. https://doi.org/10.3389/fphar.2012.00021

Venniro, M., Caprioli, D., Zhang, M., Whitaker, L.R., Zhang, S., Warren, B.L., Cifani, C., Marchant, N.J., Yizhar, O., Bossert, J.M., Chiamulera, C., Morales, M., Shaham, Y., 2017a. The Anterior Insular Cortex→Central Amygdala Glutamatergic Pathway Is Critical to Relapse after Contingency Management. Neuron 96, 414–427. https://doi.org/10.1016/j.neuron.2017.09.024

Venniro, M., Zhang, M., Shaham, Y., Caprioli, D., 2017b. Incubation of Methamphetamine but not Heroin Craving after Voluntary Abstinence in Male and Female Rats. Neuropsychopharmacology. https://doi.org/10.1038/npp.2016.287

Voorn, P., Vanderschuren, L.J.M.J., Groenewegen, H.J., Robbins, T.W., Pennartz, C.M.A., 2004. Putting a spin on the dorsal-ventral divide of the striatum. Trends Neurosci. https://doi.org/10.1016/j.tins.2004.06.006

Wang, G. Bin, Zhang, X.L., Zhao, L.Y., Sun, L.L., Wu, P., Lu, L., Shi, J., 2012. Drug-related cues exacerbate decision making and increase craving in heroin addicts at different abstinence times. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-011-2617-5

Wang, G., Shi, J., Chen, N., Xu, L., Li, J., Li, P., Sun, Y., Lu, L., 2013. Effects of Length of Abstinence on Decision-Making and Craving in Methamphetamine Abusers. PLoS One. https://doi.org/10.1371/journal.pone.0068791

Wang, J., Ishikawa, M., Yang, Y., Otaka, M., Kim, J.Y., Gardner, G.R., Stefanik, M.T., Milovanovic, M., Huang, Y.H., Hell, J.W., Wolf, M.E., Schlüter, O.M., Dong, Y., 2018. Cascades of Homeostatic Dysregulation Promote Incubation of Cocaine Craving. J. Neurosci. https://doi.org/10.1523/JNEUROSCI.3291-17.2018

Werner, C.T., Milovanovic, M., Christian, D.T., Loweth, J.A., Wolf, M.E., 2015. Response of the Ubiquitin-Proteasome System to Memory Retrieval After Extended-Access Cocaine or Saline Self-Administration. Neuropsychopharmacology 40, 3006–3014. https://doi.org/10.1038/npp.2015.156

West, E.A., Saddoris, M.P., Kerfoot, E.C., Carelli, R.M., 2014. Prelimbic and infralimbic cortical regions

differentially encode cocaine-associated stimuli and cocaine-seeking before and following abstinence. Eur. J. Neurosci. https://doi.org/10.1111/ejn.12578

Wolf, M.E., 2016. Synaptic mechanisms underlying persistent cocaine craving. Nat. Rev. Neurosci. https://doi.org/10.1038/nrn.2016.39

Wolf, M.E., Ferrario, C.R., 2010. AMPA receptor plasticity in the nucleus accumbens after repeated exposure to cocaine. Neurosci. Biobehav. Rev. https://doi.org/10.1016/j.neubiorev.2010.01.013

Wolf, M.E., Tseng, K.Y., 2012. Calcium-permeable AMPA receptors in the VTA and nucleus accumbens after cocaine exposure: when, how, and why? Front. Mol. Neurosci. https://doi.org/10.3389/fnmol.2012.00072

Wu, J., Zhu, D., Zhang, J., Li, G., Liu, Z., Sun, J., 2016. Melatonin treatment during the incubation of sensitization attenuates methamphetamine-induced locomotor sensitization and MeCP2 expression. Prog. Neuro-Psychopharmacology Biol. Psychiatry. https://doi.org/10.1016/j.pnpbp.2015.09.008

Xi, Z.X., Li, X., Li, J., Peng, X.Q., Song, R., Gaál, J., Gardner, E.L., 2013. Blockade of dopamine D3receptors in the nucleus accumbens and central amygdala inhibits incubation of cocaine craving in rats. Addict. Biol. https://doi.org/10.1111/j.1369-1600.2012.00486.x

Yu, W., Blas, A.L. De, 2008. Gephyrin expression and clustering affects the size of glutamatergic synaptic contacts. J. Neurochem. 104, 830–845. https://doi.org/10.1111/j.1471-4159.2007.05014.x

Yun, I.A., Nicola, S.M., Fields, H.L., 2004. Contrasting effects of dopamine and glutamate receptor antagonist injection in the nucleus accumbens suggest a neural mechanism underlying cue-evoked goal-directed behavior. Eur. J. Neurosci. 20, 249–263. https://doi.org/10.1111/j.1460-9568.2004.03476.x

Zhou, W., Zhang, F., Liu, H., Tang, S., Lai, M., Zhu, H., Kalivas, P.W., 2009. Effects of training and withdrawal periods on heroin seeking induced by conditioned cue in an animal of model of relapse. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-008-1414-2

Zlebnik, N.E., Carroll, M.E., 2015. Prevention of the incubation of cocaine seeking by aerobic exercise in female rats. Psychopharmacology (Berl). https://doi.org/10.1007/s00213-015-3999-6

Zséli, G., Vida, B., Szilvásy-Szabó, A., Tóth, M., Lechan, R.M., Fekete, C., 2018. Neuronal connections of the central amygdalar nucleus with refeeding-activated brain areas in rats. Brain Struct. Funct. 223, 391–414. https://doi.org/10.1007/s00429-017-1501-4