Behavioural and cellular basis of the vulnerability to develop compulsive heroin seeking habits

This dissertation is submitted for the degree of Doctor of Philosophy

Maxime Fouyssac

Downing College

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Department of Psychology

University of Cambridge

Downing Street

Cambridge

CB2 3EB

United Kingdom

Summary

PhD candidate: **Mr. Maxime Fouyssac** (Dept. of psychology)

Title: **Behavioural and cellular basis of the vulnerability to develop compulsive heroin seeking habits**

Addiction is a chronic relapsing disorder for which there is no effective treatment. This may reflect our lack of understanding of the psychological and neural mechanisms that support the transition, in vulnerable individuals, from recreational drug use to compulsive drug seeking habits. Over the last decade clinical and preclinical studies have begun to shed light on the psychological and neural basis of the individual vulnerability to cocaine addiction, but despite the epidemic in opiates addiction in the USA and incremental opioid drug abuse and addiction in the UK, heroin addiction has hitherto been under-investigated.

Using a novel preclinical model of compulsive heroin seeking behaviour in which some rats self-administering heroin persist in responding under a second-order schedule of reinforcement despite punishment (Chapter 3), the experiments in this thesis investigated the psychological, behavioural, neural and cellular mechanisms involved in the vulnerability to develop compulsive heroin seeking. Chapter 4 aimed to identify behavioural traits, such as anxiety, stress reactivity or decision making, that predict an increased vulnerability to develop compulsive heroin seeking. Chapter 5 aimed to characterise the neural and cellular correlates of heroin seeking habits, and compulsivity. Based on the combination of hotspot analysis, quantitative PCR, RNAscope and western-blot analyses, the data presented demonstrate that compulsive habits are associated with a differential pattern of cellular plasticity within corticostriatal networks, and are preceded by diverse cellular adaptations, especially in the striatum, in vulnerable individuals.

Finally, chapter 6 further investigated the cellular specificity of the observed adaptations in experiments that revealed exposure to heroin and cocaine, triggers a downregulation of the dopamine transporter preferentially in astrocytes, and not in neurons as previously thought.

The results presented in this thesis offer new insights into the neural and cellular basis of the vulnerability to develop compulsive heroin seeking, a key feature of opioid addiction.

CONTENTS

PREFACE

The following work was conducted with the Department of Pharmacology and subsequently the Department of Psychology, University of Cambridge, during the years of 2014-2017 under the supervision of Doctor David Belin.

This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration except as declared in the Preface and specified in the text.

It is not substantially the same as any that I have submitted, or, is being concurrently submitted for a degree or diploma or other qualification at the University of Cambridge or any other University or similar institution except as declared in the Preface and specified in the text. I further state that no substantial part of my dissertation has already been submitted, or, is being concurrently submitted for any such degree, diploma or other qualification at the University of Cambridge or any other University of similar institution except as declared in the Preface and specified in the text.

It does not exceed the prescribed word limit for the relevant Degree Committee.

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I now switch to Moliere’s language because some of the following persons who helped me to go through this period of my life might not be equally comfortable with the one of Shakespeare.

A ma famille, mes parents et ma sœur, je vous dois tout, merci du fond du cœur de votre

constante présence et soutien durant ces dernières années. Merci de m'avoir fait confiance et d'avoir encouragé les choix qui m'ont mené jusque-là.

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PUBLICATIONS

Publications in peer-review journals

1. Hodebourg R, Murray J, **Fouyssac M**, Puaud M, Everitt BJ & Belin D (2017) *N-acetylcysteine reduces dorsolateral striatum dopamine-dependent heroin seeking in rats*, EJN, In prep.

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3. Belin-Rauscent A, **Fouyssac M**, Bonci A & Belin D (2015) *How have preclinical models evolved to resemble addiction as defined in the DSM-5,* Biological Psychiatry 79(1). doi:10.1016/j.biopsych.2015.01.004

4. Ducret E, Puaud M, Lacoste J, Belin-Rauscent A, **Fouyssac M**, Dugast E, Murray JE, Everitt BJ, Houeto JL & Belin D (2015) *N-Acetylcysteine Facilitates Self-Imposed Abstinence After Escalation of Cocaine Intake*, Biological Psychiatry, doi:10.1016/j.biopsych.2015.09.019

Conference abstracts

1. Belin-Rauscent A., Torrisi SA., **Fouyssac M.**, Higuera-Matas A., Everitt BJ., Belin D. (2017) *Functional shift in Noradrenergic control over behaviour between the development and the expression of compulsivity*, European Behavioural Pharmacology Society (EBPS) biennial meeting, Heraklion, Crete, Greece (August 31- September 3).

2. **Fouyssac M**., Puaud M., Page G., Everitt BJ, Janet T., Belin D. (2016) *Contribution of striatal astrocytes to intrastriatal processes subserving drug seeking habits*, Cambridge Neuroscience Seminar (CNS) meeting, Cambridge, UK (17th March).

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astrocytes to intrastriatal processes subserving drug seeking habits, Federation of European Neurosciences Societies (FENS) forum of neuroscience, Copenhagen, Denmark (July 2-6).

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6. Murray JE, **Fouyssac M**., Everitt BJ., Belin D. (2015) *Differential involvement in amygdala nuclei in the acquisition and maintenance of habitual cocaine-seeking behaviour,* European Behavioural Pharmacology Society and European Brain and Behaviour Society (EBPS/EBBS) joint meeting, Verona, Italy (September 12-15).

7. Vanhille N, Ducret E., Puaud M., Ansquer S., **Fouyssac M.**, Houeto JL, Belin D. (2013), *Differential effect of environmental enrichment on the acquisition of drug use and the transition to addiction*", European Behavioural Pharmacology Society (EBPS) biennial meeting, La Rochelle, France (September 6-9).

LIST OF ABBREVIATIONS

A1	adenosinergic receptor 1
A2A	adenosinergic receptor 2A
AAV	adeno-associated virus
Acb	nucleus accumbens
AcbC	nucleus accumbens core
AcbS	nucleus accumbens shell
aDLS	anterior dorsolateral striatum
aDMS	anterior dorsomedial striatum
AI	agranular insular cortex
AL	active lever
ANOVA	analysis of variance
A-O	action-outcome
Astro	astrocytes
AWERB	animal welfare and ethical review body
BDM	bad decision makers
bFGF	basic fibroblast growth factor
BLA	basolateral amygdala
CB1	cannabinoid receptor 1
cDNA	complementary deoxyribonucleic acid
CeA / CeN	central nucleus of the amygdala
COMT	catechol-o-methyltransferase
CPP	conditioned place preference
CR	conditioned reinforcer
CRs	conditioned reinforcers

CS	conditioned stimulus
CSs	conditioned stimuli
Ct	cycle threshold
D1	dopamine receptor 1
D2	dopamine receptor 2
D3	dopamine receptor 3
DAPI	diamidino-2-phenylindole
DAT	dopamine transporter
DI	disgranular insular cortex
DLS	dorsolateral striatum
DMEM	Dubelcco's modified Eagle's medium
DMS	dorsomedial striatum
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DOPAC	dihydroxyphenylacetic
DSM	diagnostic and statistical manual of mental disorders
DYN	dynorphin
ECL	electrochemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGR1	early growth response 1
EPM	elevated plus maze
FACS	fluorescent-activated cell sorting
FI	fixed interval
FR	fixed ratio
GABA	gamma-aminobutyric acid
GDM	good decision makers

GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GLT-1	glutamate transporter 1
GMM	general materials and methods
GT	goal trackers
HA	high anxious
HC	high compulsive
HNP	high novelty preference
HP	high preference for saccharin
HR	high responders
HRP	horseradish peroxidase
HVA	homovanillic acid
IBA-1	ionised calcium binding adaptor molecule 1
IC	intermediate compulsive
IL	inactive lever / infralimbic cortex
ISH	in situ hybridisation
IU	international unit
Kappa	kappa-opioid receptor
LA	low anxious
l-AcbS	lateral accumbens shell
Lat BLA	lateral basolateral amygdala
LC	low compulsive
LgA	long access
LNP	low novelty preference
lOFC	lateral orbitofrontal cortex
LP	low preference for saccharin

LR	low responders
m-AcbS	medial accumbens shell
MAM	monoacetylmorphine
MAO	monoamine oxidase
mOFC	medial orbitofrontal cortex
mRNA	messenger ribonucleic acid
Mu	mu-opioid receptor
NAC	N-acetylcysteine
NCBI	national center for biotechnology information
NET	norepinephrine transporter
NeuN-1	neuronal nuclei 1
NS	non-significant
OA	open arms
OD	optical density
OP	open field
PBS	phosphate buffer saline
PCR	polymerase chain reaction
pDLS	posterior dorsolateral striatum
pDMS	posterior dorsomedial striatum
PFA	paraformaldehyde
PFC	prefrontal cortex
pH	potential of hydrogen
PL	prelimbic cortex
PR	progressive ratio
qPCR	quantitative polymerase chain reaction
RGT	rat gambling task

RMTg	rostromedial tegmental nucleus
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature / reverse transcription
SA	self-administration
Sacc	saccharine
SERT	serotonin transporter
ShA	short access
SNc	substantia nigra pars compacta
SOR	second order schedule of reinforcement
S-R	stimulus-response
SSC	saline sodium citrate
ST	sign trackers
TdT	terminal deoxynucleotidyl transferase
TE	tris ethylenediaminetetraacetic acid
tRNA	transfer ribonucleic acid
US	unconditioned stimulus
VI	variable interval
VMAT	vesicular monoamine transporter
vOFC	ventral orbitofrontal cortex
VR	variable ratio
Vs	versus
VTA	ventral tegmental area
xCT	cysteine/glutamate antiporter

CHAPTER 1: GENERAL INTRODUCTION

Addictive drugs: a social and health problem

Drug use and addiction represent a heavy burden for modern societies in terms of both economic and social costs. A large number of psychoactive substances (legal or illegal) have been classified as drugs of abuse and their chronic consumption can lead to an addiction in those individuals who are vulnerable to lose control over their recreational use [11]. The so-called "hazardous nature" of a drug of abuse is not related to its legal status, but it can be defined by both its dependence/addictive properties and its consequences on the physical and psychological health of the user. According to these criteria, heroin and cocaine, which will be the focus of the present work, are the most dangerous drugs [10, 12] (**Fig. 1.1**).

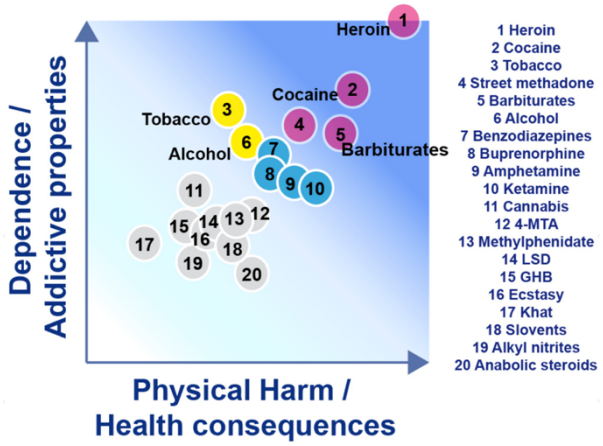


Figure 1.1: Scale assessing the "hazardous" nature of psycho-active substances. *Correlation between the addictive property of a substance and the consequences of its consumption. The most dangerous ones appear to be heroin, cocaine, barbiturates and street methadone. Interestingly, legal drugs such as tobacco and alcohol are by far not the less dangerous ones. (adapted from[10])*

Addictive drugs have been used by our species at least since it has been structured in societies. Individuals engage in drug use for various reasons that are not necessarily related to their addictive potential.

Why do people take drugs?

A large proportion of the population experiences drugs of abuse at least once in their lifetime. The first contact with a drug of abuse is triggered either by curiosity, peer pressure, risk taking or as a self-medication strategy to cope with emotional/internal distress, such as anxiety or depression (see below). A large majority of the users can maintain control over drug intake and

keep using theirs drugs recreationally over years.

Drugs of abuse provide a broad spectrum of psychological and systemic effects associated with their very different mechanisms of action. Nevertheless, addictive drugs share common characteristics, the most important of which is their ability to increase the extracellular concentration of dopamine in the nucleus accumbens (Acb) [13], which has been suggested to be the neurochemical basis of their reinforcing properties.

Thus, addictive drugs are powerful positive reinforcers, thereby facilitating the behavioural repertoire associated to their use in recreational users. A positive reinforcer is an unconditioned stimulus (US) that increases the probability of expression of a behaviour, or response, the consequence of which is a presentation of that stimulus [14]. Behaviourally, drugs of abuse, support instrumental learning as measured in self- administration procedures. Under continuous reinforcement, or other fixed ratio schedules of reinforcement (detailed below), which are used to investigate the reinforcing properties of drugs, animals quickly learn the contingency between an instrumental response (a behaviour that does not belong to the behavioural repertoire of the species, e.g. a lever press) and the intravenous delivery of a drug, and titrate their intake so as to reach and maintain an optimal level of reinforcement [15].

It has been suggested that the reinforcing properties of drugs primarily depend on the Acb shell and the influence of their associated cues on instrumental responding, on the core of the nucleus accumbens (AcbC) [16]. Thus, the AcbC plays a critical role in mediating the link between the motivational and sensory-specific properties of stimuli modulated by reward expectation [17-20] and instrumental responses. It has long been considered to be a “limbic-motor interface” by which motivation or Pavlovian mechanisms influence reward-driven instrumental performance [21]. However, AcbC neurons are also activated in response to novelty and their activation enables the comparison of the predicted rewarding value of a stimulus to its current subjective value [22], which is a key mechanism underlying the incentive learning and decision making functions of dopamine [23, 24]. The AcbC receives glutamatergic projections from the prefrontal cortex, the hippocampus and the basolateral amygdala (BLA) while its dopaminergic inputs originate in the ventral tegmental area (VTA). AcbC sends GABAergic projections [25] to the Globus pallidus, the VTA, and the substantia nigra (**Fig.1.2**), among other structures, thereby

interfacing emotions and actions.

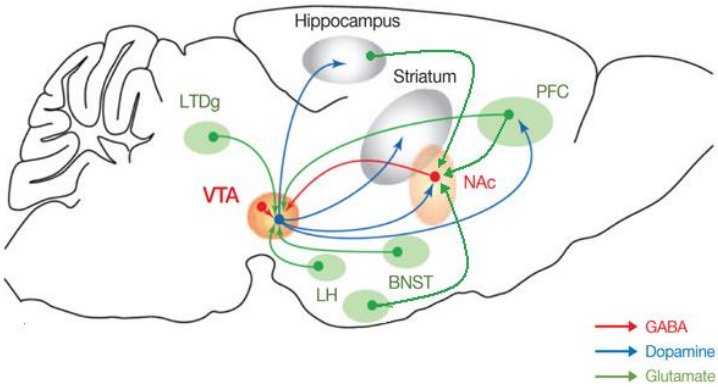


Figure 1.2: Schematic representation of the mesolimbic system within a broader motivation circuit. *Glutamatergic afferents (green) excite post-synaptic neurons while GABAergic afferents (red) inhibit them. The release of dopamine by dopaminergic neurons exhibits complex modulatory effects, mediated by the g-proteins to which are coupled the post-synaptic dopamine receptors. Drugs of abuse increase dopamine concentration in the mesolimbic system. VTA, Ventral Tegmental Area ; Nac, Nucleus Accumbens Core ; AMG, Amygdala ; BNST, Bed Nucleus of the Stria Terminalis ; LH, Lateral Hypothalamus ; PFC, Pre Frontal Cortex ; LTDg, Lateral Dorsal Tegmental Nucleus.(adapted from [8]).*

At the cellular level, dopaminergic neurons fire in response to unexpected stimuli in the environment either new or after violation of a prediction [26], and offer a teaching signal to the motivational system by which they facilitate associative and instrumental learning and contribute to ascribe incentive value to cues. Thus, dopaminergic neurons offer a cellular mechanism supporting a prediction error learning system in the brain [26]. Their firing is controlled by local GABAergic interneurons in the VTA or GABAergic neurons from the rostromedial tegmental nucleus (RMTg) which are characterised by their high level of expression of the molecular target of morphine, the mu-opiate receptor (μ-OR). When an action potential reaches their terminals, a sudden increase in calcium concentration triggers the release of dopamine into the synaptic cleft. Dopamine activates its pharmacological targets, the metabotropic D1 and D2-like receptors, at the membrane of post-synaptic neurons and D2 receptors on pre-synaptic neurons. The termination of the dopaminergic signal is governed par two mechanisms: a specific reuptake of dopamine via the DAT [27] both expressed in neurones and astrocytes [28], and an enzymatic degradation of dopamine by the monoamine oxidases (MAO) and the catechol-o-methyltransferase (COMT) that are also expressed in neurones and astrocytes [29, 30] and also located within the synaptic cleft. Neuronal and astrocytic DAT remains the primarily mechanism of the termination of dopamine transmission and is efficient enough that the presynaptic pool of dopamine stems from reuptake of dopamine more than new synthesis (**Fig. 1.3 A**).

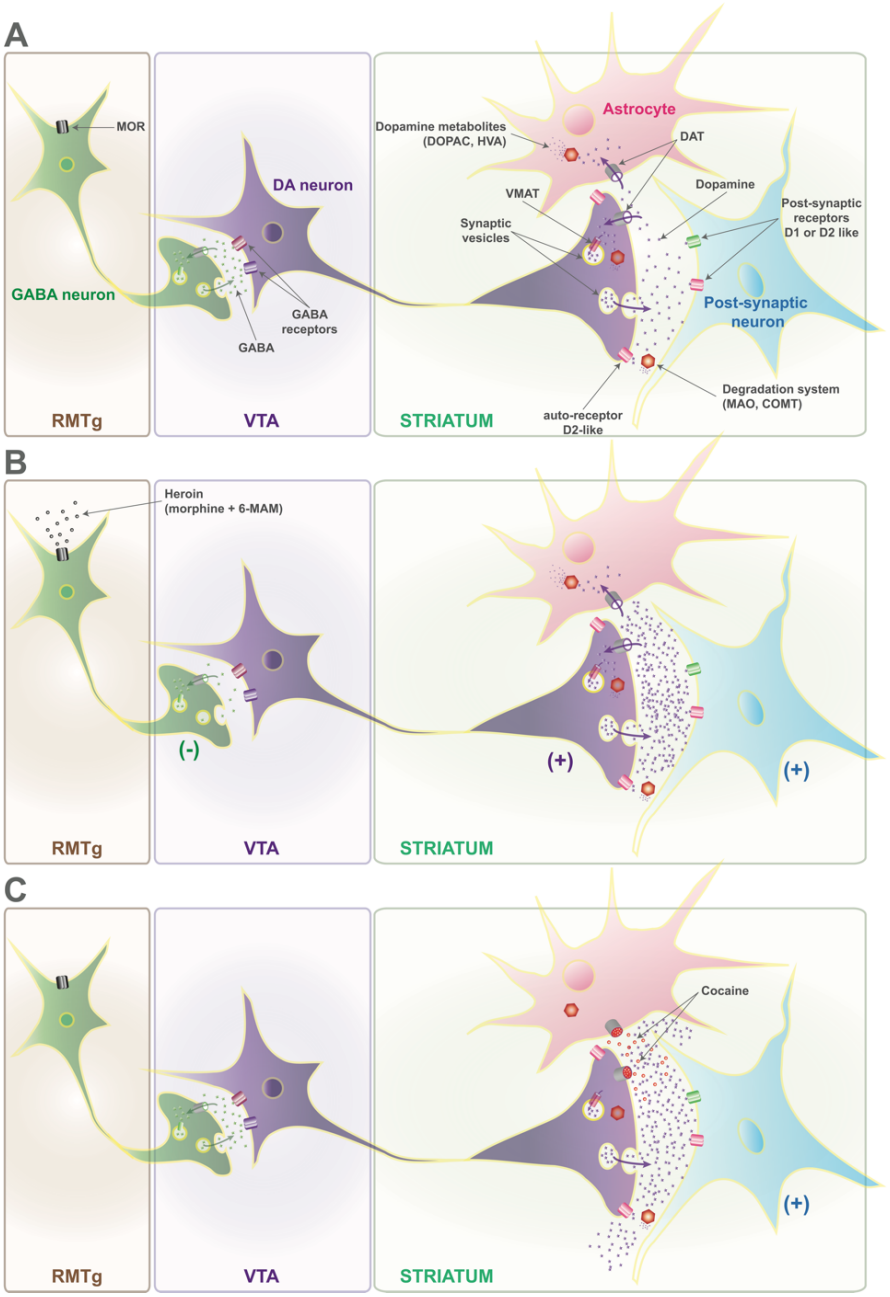


Figure 1.3: Mechanisms of drug-induced enhancement of dopaminergic signalling in the ventral striatum. ***A)*** *Drug-free physiological activity of dopamine synapses in the ventral striatum. In dopaminergic neurons, dopamine (purple dots) is transported into vesicles by VMAT. Upon depolarisation of the pre-synaptic terminal by an action potential, a calcium-dependent release of dopamine into the synaptic cleft occurs following exocytosis of the vesicle content. Dopamine activates its pharmacological targets (i.e. D1- and D2-like receptors) at the membrane of post-synaptic and pre-synaptic neurons (only D2). The termination of dopamine signalling is driven by two mechanisms: reuptake by DAT expressed at the membrane both of pre-synaptic neurons and astrocytes, and enzymatic degradation by COMT and MAO into metabolites (DOPAC, HVA) expressed on neurons and astrocytes but also located within the synaptic cleft. Dopaminergic neurons, whose cell bodies are located in the VTA, are modulated by GABAergic neurons located in the RMTg as well as the VTA. These neurons exert an inhibitory tonus on dopaminergic neurons mediated by GABAa receptors.* ***B)*** *Heroin influence over dopaminergic synapses in the ventral striatum. Interaction between metabolites of heroin and μ-OR inhibits GABAergic neurons, resulting in a desinhibition of dopamine neurons. Dopaminergic neurons fire more, in response to stimuli, leading to a hyperdopaminergic state of the striatal synapses.* ***C)*** *Cocaine-induced alterations of dopamine synapses in the ventral striatum. Cocaine (red dots) inhibits dopamine reuptake by blocking DAT, which leads to a hyperdopaminergic state at the striatal synapses as well as the somatodendritic compartment in the mesencephalon.. [MOR, mu-opioid receptors; RMTg, rostromedial tegmental nucleus; VTA, ventral tegmental area; DA, dopamine; DAT, dopamine active transporter; VMAT, vesicular monoamine transporter; DOPAC, dihydroxyphenylacetic acid; HVA, homovanilic acide; MAO, monoamine oxydase; COMT, catechol-O-methyl transferase; 6-MAM, 6- monoacetylmorphin].*

Astrocytes represent a subpopulation of glial cells whose function has long been considered to be restricted to a "basic" supportive role towards central nervous system (CNS) homeostasis. Thus, astrocytes contribute to control cerebral blood flow [31], extracellular pH [32], potassium buffering [33, 34] or mediate the exchange of gases through facilitation of water transport [35]. In particular, astrocytes have been functionally studied for their pivotal role in bridging metabolically neurons to vascular glucose, thereby providing neurons with the energy "fuel" they need to sustain their energy-demanding activity [36].

However, over the last decade, a wealth of evidence has challenged this restrictive view of the function of astrocytes and has suggested a broader and more complex role for these cells in the central nervous system. Astrocytes are increasingly being considered as key players in the regulation of synaptic activity and plasticity and associated behavioural and psychological functions. Even what was considered merely a basic energetic supply provided to neurons by neighbour astrocytes, namely lactate, has recently been shown to modulate synaptic activity and to directly contribute to cocaine-associated appetitive Pavlovian mechanisms [37, 38].

As previously stated, cocaine and heroin both increase the extracellular concentration of dopamine via different mechanisms of action. Cocaine blocks DAT activity, thereby enhancing extracellular concentration of dopamine by impairing neurotransmitter clearance from the synaptic cleft, and thus in an activity-dependent manner. On the other hand, heroin triggers a disinhibition of dopamine neurons and an associated hyperdopaminergic state at synapses in an activity-independent manner.

Cocaine (benzoylmethylecgonine), an alkaloid found in coca leaves, is a psychostimulant. It inhibits monoamine reuptake from the synaptic clefts. In the central nervous system, the more potent psychostimulant effect of cocaine is attributable to the inhibition of the dopamine active transporter "DAT" (Ki ≈ 0.23µM) [39] by stabilizing the outward-facing conformation of the transporter [40]. It binds to the DAT at the same site as dopamine and blocks its reuptake at dopaminergic synapses (**Fig. 1.3 C**). However, cocaine has also some affinities for the serotonin transporter "SERT" (Ki ≈ 0.74µM), for the noradrenalin transporter "NET" (Ki ≈ 0.48µM) [39], for Sigma-1, muscarinic M1 and M2 acetylcholine receptors [41, 42] and for the alpha subunits of sodium channels (type 5, 11 and 10) [43-45].

Heroin (diacetylmorphine) is synthesized from morphine that is naturally found in opium poppy. In the body plasma, heroin is hydrolysed in three metabolites: 6-monoacetylmorphine (6-MAM), morphine and 3-monoacetylmorphine (3-MAM) [46]. The pharmacologically active metabolites of heroin are 6-MAM and morphine [47] and act as agonists for the 3 categories of opioid receptors that are mu-, kappa-, and delta-opioid receptors (respectively known as μ-OR, κ-OR and δ-OR) [48-50] (**Fig.1.4 A**). However, 6-MAM crosses the blood brain barrier more quickly than morphine, such as activation of MOR by 6-MAM is responsible for the quick sense of, or high, euphoria that is not observed following morphine consumption [51]. The μ-OR is a Gi/Go protein coupled metabotropic receptor [52] that mediates the rewarding properties of heroin [53, 54] (**Fig. 1.4 B**). Its activation in the mesocorticolimbic system, and more particularly in the rostro medial tegmental nucleus (RMTg, also known as the "tail of the VTA"), reduces the firing of GABAergic neurons that leads to a disinhibition of the dopaminergic neurons in the VTA, thereby resulting in an increase in dopamine transmission in the Acb [55-58] (**Fig. 1.3 B**).

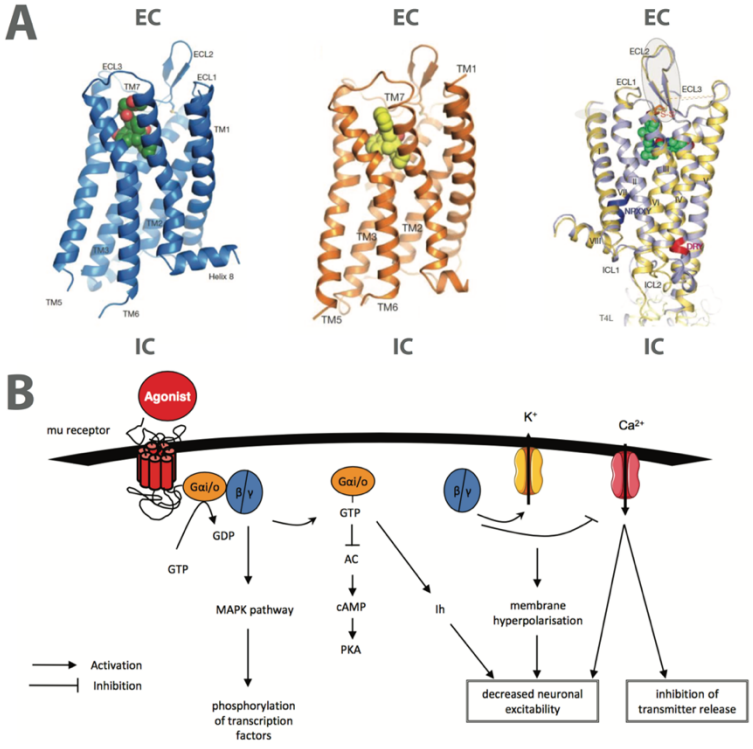


Figure 1.4: Architecture of the opioid receptors and signal transduction induced by MOR activation. ***A****) Views from within the membrane plane show the typical seven transmembrane domains G protein coupled receptor architecture of MOR, DOR and KOR (adapted from [1-3]).* ***B****) Ligand-induced MOR activation leads to an inhibition of neurotransmitter release and a decrease in neuronal excitability by promoting K+ release and inhibiting the entrance of Ca2+ (adapted from [4]). EC, extracellular; IC, intracellular; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; Ih, voltage-dependant current; MAPK, mitogen-activated protein kinase; PKA, protein kinase A.*

Consequently, cocaine and heroin do not similarly influence the activity of dopaminergic neurons. Dopaminergic neurons exhibit a basal activity which gives rise to a "tonic" concentration of the neurotransmitter at the synaptic cleft (from 5 to 20nM) [59], while once neurons are stimulated, the induced burst firing gives rise to a "phasic" level of dopamine (up to 1µM) [60]. This "phasic" dopamine signal is thought to be responsible of the ability of dopamine neurons projecting to topographically overlapping sites, to fire synchronously and to support their role in incentive learning [23, 61]. Heroin mostly affects "tonic" dopamine transmission while it has been shown that inhibition of DAT activity enhances both "phasic" and "tonic" dopamine signals [62].

Thus, the influence of exposure to cocaine or heroin on the function of the astrocytic DAT, which should contribute to these adaptations in the physiology of dopamine transmission in the striatum, remain to be elucidated.

Acute and repetitive exposures to addictive drugs therefore alter the structure and function of the mesolimbic system, including inducing aberrant plasticity [63]. However, chronic exposure to the drugs results in additional neurobiological adaptations that encompass other striatal and cortical structures, which have been shown, in humans as well as in non-human primates and rodents self-administrating drugs, to be related with addictive states. Importantly, each individual exposed to drugs eventually undergoes these adaptations and the reason why only a fraction of these users develop addiction remains unknown. It is therefore important to consider the notion of individual vulnerability when trying to understand the psychobiological basis of addiction.

Indeed, a large majority of users are able to maintain a control over drug intake while 15 to 30% of them (depending on the drug) will eventually develop addiction [11, 64] (**Fig.1.5**). These vulnerable individuals not only take a lot of drugs, but they spend a great deal of time foraging for these drugs, under the control of conditioned stimuli in the environment. Thus, addiction has been defined as a chronic relapsing mental disorder characterised by a compulsive drug seeking and taking behaviour [65], a loss of control over drug intake, and the associated emergence of a negative emotional state reflected at withdrawal [66].

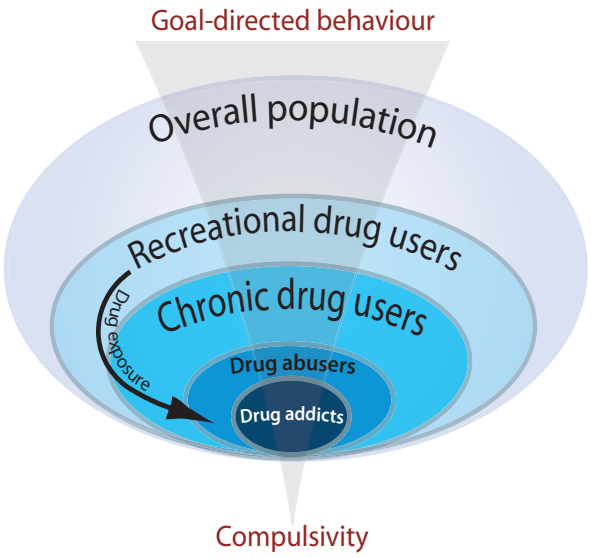


Figure 1.5: Inter-individual differences in the vulnerability to develop drug addiction. *Among the overall population, a large majority of individuals will be able to maintain control over their drug intake while 15 to 30% of users will develop drug abuse and, eventually, addiction. Drug addicts seek and take drugs compulsively: they maintain drug use despite awareness of adverse consequences (social, health, and professional). The development of drug addiction is underlined by a progressive transition from a goal-directed taking/seeking behaviour to a compulsive habit (adapted from [7])*

Beyond reinforcement: why do people eventually lose control?

Over the last decades, human studies revealed some behavioural impairments inherent to drug abuse whereby addicts display deficits in inhibitory control [67-75], insight [76-78] and decision making [78-85]. Addiction has also been associated with comorbid psychiatric disorders such as psychosis or depression and post-traumatic stress disorder, and distinct behavioural/personality traits including anxiety, [86-92], impulsivity and sensation seeking [93-95].

While, these behavioural and cognitive factors have been associated with addiction to various drugs of abuse, i.e. psychostimulants, opiates, alcohol or cannabis, the extent of the association is somehow predicated on the nature of the drug used, indeed, heroin addicts display greater anxiety [96] but lower levels of impulsivity [67, 97, 98] than cocaine/crack addicts.

It remains unknown whether these cognitive deficits, behavioural personality traits and comorbid factors are a consequence of chronic drug use or whether they predate drug use and therefore represent factors of vulnerability to drug addiction. They could also represent factors of increased propensity to initiate drug use, as it has been suggested for anxiety and sensation seeking [91, 92, 99-104]. The onset of drug use seems to be also under the influence of distinct pre-existing psychoaffective states [105, 106] whereby individuals start taking drugs as a self-

medication strategy. In this self-medication hypothesis of addiction [107-110], drug intake is supported from the onset by negative, rather than positive reinforcement, and may well facilitate the emergence of compulsivity as suggested by Koob and Le Moal [111].

The architecture of personality is shaped and structured by interaction with the environment throughout the neurodevelopmental stages [112]. In this context, poor life conditions as those experienced in deteriorated living areas, child abuse or pathological families may shape the construction of a personality conferring an increased vulnerability to drug addiction [113]. In contrast, positive relationships within the family and the community seem to be protective against drug use [114].

Imaging studies, have further revealed that the aforementioned behavioural impairments displayed by drug addicts are associated with alterations of both brain structure and function. At the structural level, drug addicts have been shown to display reduced grey matter volume in cerebellar [115] and prefrontal areas [116-120].

Functionally, the most reported alterations associated with drug addiction have been shown to involve the fronto-striatal systems suggested to support the compulsive nature of drug abuse [121]. When presented drug-related cues that increase self-reported craving [122, 123], individuals with an addiction show functional abnormalities in the frontal areas such as the ventromedial and dorsolateral prefrontal cortices [124], orbitofrontal cortices [125, 126], cingulate cortex [126, 127] as well as the insular cortex [76, 128] and the amygdala [129, 130]. Presentation of drug-related cues not only recruit the limbic system but also dopamine release in the dorsal territories of striatum (the neural locus of control over habits) of individuals addicted to drugs [131, 132] as well as recreational drug users who do not meet the DSM criteria for addiction [133]. As stated by Ersche and colleagues, we are still facing the “chicken-and-egg dilemma” [134] in so that it remains unknown whether these functional abnormalities predate or are a consequence of drug use.

At the molecular level, addiction to opiates and psychostimulants has been associated with a diminished level/availability of striatal dopamine 2 and 3 receptors [121, 135-137] which has been related to a hypofunction of the orbitofrontal cortex [135]. Even if addiction to opiates and psychostimulants share some common structural and functional alterations, human studies

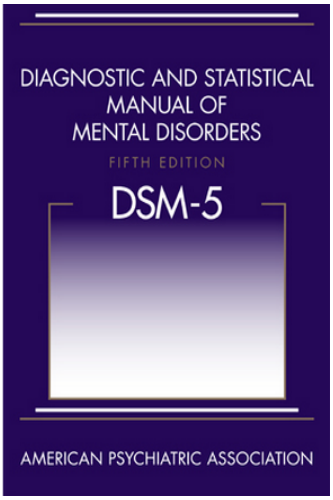
revealed some major differences in the genetic profiles individuals addicted to these two drugs, as described by Kreek and colleagues [9] (**Table 1.1**). Yet these genetic determinants account for a very small proportion of the total variance.

Gene	Protein	Drug
OPRM1	mu opioid receptor	h + c
OPRK1	kappa opioid receptor	h
OPRD1	delta opioid receptor	h + c
PDYN	prodynorphin	c
PENK	proenkephalin	h + c
POMC	proopiomelanocortin	h + c
HOMER1	homer homolog 1	c
TACR3	tachykinin receptor 3	c
MC2R	melanocortin receptor type 2 (ACTH receptor)	h
DRD2	dopamine receptors D2	h + c
DRD4	dopamine receptors D4	h + c
SLC6A3	dopamine transporter 1 (DAT1)	c
COMT	catechol-O-methyltransferase	h + c
HTR1B	serotonin receptor 1B	h
HTR3B	serotonin receptor 3B	h
SLC6A4	serotonin transporter (SERT)	h
TPH2	tryptophan hydroxylase 2	h
GABRG2	GABAA receptor gamma 2	h
CNR1	cannabinoid receptor 1	h + c
CHRM2	cholinergic muscarinic 2 receptor	h + c
BDNF	brain-derived neurotrophic factor	h + c
PER3	period circadian protein 3	h
CSNK1E	casein kinase 1 epsilon	h
GAL	galanin	h
ADH	alcohol dehydrogenase gene cluster	h + c

Table 1.1: Genes associated with heroin and/or cocaine addiction by hypothesis-driven single or multiple gene association studies. *Several studies reported association of cocaine addiction and/or heroin addiction with genetic variants reflecting the common and differential polymorphisms associated with cocaine or heroin addiction. [h=heroin, C=cocaine] (adapted from [9])*

Together, genetic factors might contribute to up to 40% of the vulnerability to develop addiction [138] but It remains unknown whether these genetic determinant are associated with an increased propensity to engage in drug use or indeed the vulnerability to addiction. In addition, the inter-individual variability observed in drug addicts among the population, in their pattern of drug intake, the frequency of intoxication, the consumption of other drugs, their environment or the expression of co-morbid psychiatric disorders, render human studies difficult to interpret sometimes.

Thus, understanding the psychological, neural and cellular basis of this inter-individual vulnerability to develop addiction has been, and remains, a real challenge, especially since the clinical definition of the disorder, as stated in the DSM, has evolved dramatically since 1987, eventually to offer a dimensional perspective in the last iteration published in 2015 [139] (**Table 1.2**).



Category	Criteria
Impaired control	• Opioids used in larger amounts or for longer than intended • Unsuccessful efforts or desire to cut back or control opioid use • Excessive amount of time spent obtaining, using, or recovering from opioids • Craving to use opioids
Social impairment	• Failure to fulfill major role obligations at work, school, or home as a result of recurrent opioid use • Persistent or recurrent social or interpersonal problems that are exacerbated by opioids or continued use of opioids despite these problems • Reduced or given up important social, occupational, or recreational activities because of opioid use
Risky use	• Opioid use in physically hazardous situations • Continued opioid use despite knowledge of persistent physical or psychological problem that is likely caused by opioid use
Pharmacological properties	• Tolerance as demonstrated by increased amounts of opioids needed to achieve desired effect; diminished effect with continued use of the same amount • Withdrawal as demonstrated by symptoms of opioid withdrawal syndrome; opioids taken to relieve or avoid withdrawal

Table 1.2: Summarized DSM-5 diagnostic categories and criteria for opioid use disorder (adapted from [6]).

Addiction: clinical definition and psychological constructs

Based on the *diagnostic and statistical manual of mental disorders* [139], substance use disorder is clinically characterised by a pathological pattern of behaviours associated with the use of the substance. Thus, clinically, drug addiction is defined by the expression of at least two of the following impairments occurring within a 12-month period (**Table 1.2**). This symptomatic multi-dimensional approach is very helpful for establishing a diagnosis but it does not offer an answer to the most important question: Why do people compulsively take drugs?

Despite extensive research in clinical and preclinical fields, we are yet to offer a good answer to this key question. Nevertheless, several theories have proposed psychological and neural models of addiction, namely, the incentive-sensitisation, the hedonic-allostasis and the maladaptive habit theories.

Main theories of addiction: towards a unitary view?

The three major theories of addiction mentioned above have been developed to attempt to capture the psychological and neural basis of addiction. Potentially accounting for different stages or contributing factors of the addiction process, these theories are not mutually exclusive, but they all have received independent experimental support from different animal models of

addiction that will be briefly discussed later in this chapter.

There are several key facts to capture in any successful theory of addiction; it is of course possible that no one theory will accommodate all forms of drug addiction. The main consideration that has long been the focus of addiction research is that addiction follows the chronic use and abuse of drugs, and so is more to do with the adaptations (including conditioning) that the brain makes to repeated drug experiences, rather than to the drug's initial reinforcing effects and primary site of action. Thus, the three theories discussed here involve the hijacking of the dopaminergic system by addictive drugs and the alteration of the associated functions including Pavlovian and instrumental learning, incentive salience and motivation as well as hedonic states.

Chiefly, the incentive sensitisation theory developed by Robinson and Berridge [140] suggests that addiction results from too much motivation for the drug, triggered by its associated cues that are imbued with incentive motivational properties. The hedonic allostasis theory, developed by Koob and le Moal [111, 141] suggests that the recruitment of the stress system in the brain in response to chronic exposure to addictive drugs triggers a negative state that contributes to a transition from positive to negative reinforcement in the pursuit of drug use. These two theories are built on the assumption that drug seeking and taking remain a goal-directed behaviour and that addicts maintain a clear representation of their goal, even if the motivational system can be divorced from the subjective appraisal of the drug, as suggested by the incentive sensitisation theory. In contrast, the maladaptive habit theory developed by Robbins and Everitt [65, 142] builds onto the effects of addictive drugs both on associative mechanisms (the focus of the incentive sensitisation theory) and instrumental mechanisms to suggest that drugs aberrantly engage stimulus-response (S-R) habitual control over drug seeking behaviour. The rigidity of the cognitive schemata triggered under the control of S-R associations is, according to this theory, rigid enough to pave the way for the loss of executive control over behaviour that characterises compulsivity.

The incentive-sensitisation theory of addiction, developed by Robinson and Berridge, focus on the motivational effects of addictive drugs. Thus, by hijacking learning mechanisms dependent of the mesolimbic dopaminergic system (describe below), drugs are suggested to aberrantly

attribute incentive salience to the conditioned stimuli (CSs) which leads to an increased desire or craving for the drugs [140]. A sensitisation of the dopamine system is suggested to occur in response to repeated exposure to addictive (namely stimulant) drugs. This theory suggests that the mechanisms supporting incentive salience that are responsible for craving are independent of those supporting the subjective pleasurable effects of the drugs [140], whereby "wanting" becomes artificially dissociated from "liking".

Koob and Le Moal developed the hedonic-allostasis theory [111], originating from the opponent processes theory of motivation [143], whereby drug use is initially motivated by the rewarding properties of the drug but progressively switches to negative reinforcement. Thus, chronic exposure triggers adaptations which lead to a decrease of the rewarding effects of the drug and the emergence of physical and psychological withdrawal symptoms. The emotional distress brought about by the latter contribute to an "hedonic allostatic state", stated by the authors to *"represent a chronic deviation of reward set point and is fuelled not only by dysregulation of reward circuits per se, but also by the activation of brain and hormonal stress responses"*[111].

The maladaptive habit theory has recently evolved into an incentive habit theory by Belin and colleagues [7, 144, 145] that proposes that addiction results from an aberrant coupling between the motivational Pavlovian mechanisms underlying the reinforcing properties of CSs when used as conditioned reinforcers and the stimulus-response associations that support drug seeking habits [146]. Thus, non-explicit impulses generated in the amygdala in response to exteroceptive or interoceptive conditioned stimuli recruit rigid drug seeking habits dependent upon the anterior DLS, thereby hijacking top down executive control mechanisms from the prefrontal cortex. This incentive habit theory offers a unique framework within which, are brought together all the mechanisms on which the aforementioned theories focus.

These theories of addiction have had a marked influence in drug addiction research over the past decades. However, they still fail to account for the inter-individual vulnerability to develop drug addiction. Kreek and colleagues offered a three-dimensional approach whereby the vulnerability to develop drug addiction is suggested to result from interactions between genetic factors, the environment and the neurobiological consequences of drug exposure [138]. The

challenge for both clinical and preclinical research is to understand the psychological and neural mechanisms whereby these interactions eventually lead to addiction.

Disentangling the contribution of these factors to the vulnerability to addiction has proven a rather difficult task in human. However, preclinical research enables longitudinal studies, within which, pre-existing personality traits and subsequent drug-induced neurobiological adaptations are assessed in a controlled-environment.

Preclinical models of addiction

The most difficult, but certainly the most important aspect of preclinical research, especially in the context of psychiatric disorders such as drug addiction is to achieve the best operationalisation of the neurobehavioural constructs or symptoms of a human condition. It is obviously impossible to encapsulate in a lower species the multi-facetted "symptoms" and characteristics of the pathology. However, a variety of preclinical models of addiction have been developed over the last decades trying to capture and operationalise some of the behavioural features of drug addiction. Since these models offer a unique opportunity to investigate the neural and cellular bases of behavioural responses to addictive drugs, the most simplistic ones have often benefited from a huge popularity in our field that has for long focused on the neurobiological adaptations to drug exposure in non-human primates and rodents. It has been considered that understanding these adaptations at the circuit, neural or synaptic level is the key to understand addiction. I would argue that this is not enough and preclinical models should be further refined to better encapsulate the neurobehavioural and psychological constructs of the disorder prior to capitalize on drug-induced neurobiological adaptations.

Yet, current approaches combining refined models of addiction with causal manipulations of the brain or investigations of the cellular and molecular mechanisms involved in drug seeking and taking behaviour, have offered better understanding of the neurobiological substrates subserving some dimensions of drug addiction. However, to better capture the complex drug-related adaptions and more importantly, the brain mechanisms underlying the vulnerability to develop addiction, it is important to move away from the "drug-centered" view of addiction supporting the popularity of experimenter-delivered injections and short-term self-

administration experiments.

1. Psychomotor sensitization

One such experimenter-delivered injections based model is the psychomotor sensitization model. This model has been extensively used as experimental support for the incentive sensitization theory. The observation that the locomotor response to intermittent experimenter-delivered drug infusions increases over time has led the community to suggest that it was a behavioural proxy for the sensitization of the incentive properties of drugs. This phenomenon is subserved by neurochemical sensitization whereby dopamine release in response to the drug increases over time [140] and many specific brain adaptations which have been suggested to facilitate psychostimulant self-administration [147, 148] and its reinstatement following extinction [149, 150]. Interestingly, it has been shown that a single injection of psychostimulants or opiates can trigger a long-lasting locomotor sensitization [151-155], suggesting that this behavioural response, which is context-dependent and does not occur following self-administration of drugs, may contribute to the early stages of drug addiction.

2. Conditioned place preference (CPP)

CPP relies on a contextual Pavlovian conditioning. It has been widely used to probe the neurobiological and psychological substrates of the “rewarding” or associative properties of several drugs of abuse, including psychostimulants and opiates [156-160] as well as those of the emergence of negative emotional state associated with withdrawal [161-163]. In this procedure, two distinct contexts (compartments of a CPP box described in General Materials and Methods (GMM)) are paired with different unconditioned stimulus (colour, odour, configuration or texture of the floor/walls). The protocol is articulated around two consecutive phases, namely conditioning and testing phase. During the conditioning phase, lasting several consecutive days, rats are injected with the drug in one of the two compartments and with a vehicle solution in the other one. On the single testing session, rats do not receive any injection and are given access to the two compartments. Preference for the drug-related one is therefore considered as CPP and reflects the “rewarding” properties of the drug.

These two models do not capture the instrumental aspect of drug seeking/taking behaviour

as displayed by rats trained to self-administer drug in operant boxes and fail to factor in the volitional nature of the initiation of drug use.

3. Drug self-administration procedures

As mentioned before, drugs of abuse act as positive reinforcers which support the acquisition of an instrumental association whereby a lever press or a nose poke is associated with the delivery of the drug. Acquisition of drug self-administration has been shown behaviourally to reflect the reinforcing properties of the drug [64]. Drug self-administration methodology was firstly described in the rat in the 1960's by Weeks [164] in a seminal experiment which freely moving rats were trained to self-administer morphine under a fixed ratio (FR) schedule of reinforcement, i.e. the drug is delivered following completion of a fixed number of responses. However, such a schedule only offers measurements of the reinforcing properties of the drugs. Thus, several schedules of reinforcement have been implemented since, to probe mechanisms supporting either drug seeking or taking behaviour, motivation and compulsivity [165]. The fixed interval (FI) schedules of reinforcement enable the quantification of seeking behaviour, whereby the drug is delivered only after completion of an instrumental response following a drug-free fixed interval of time, irrespective of the vigor of responses during the drug-free periods. Other schedules of reinforcement, which weaken the contingency between action and outcome are called variable interval (VI) and variable ratio (VR) schedules of reinforcement [165], under which, respectively, the drug-free periods or the number of responses necessary for the delivery of the drug follow an unpredictable pattern.

Over the past fifty years, different preclinical models of addiction have been developed utilising the drug self-administration procedure which successfully operationalise some psychological processes subserving the development of distinct components of drug addiction.

a. Relapse / craving

One of the behavioural hallmarks of drug addiction is the propensity individuals show to relapse after periods of abstinence [90]. This phenomenon can be operationalise for psychostimulants and opiates in preclinical research by either the reinstatement of extinguished self-administration procedure [166, 167], the forced-abstinence [168] or self-abstinence and

relapse models [169-171].

In the first model, rats are trained to self-administer a drug for a short period of time under continuous reinforcement and are, subsequently, subjected to several consecutive extinction sessions that result in a decrease in responding on the active lever. Subsequently, instrumental responding is reinstated either by an injection of the drug (drug-induced reinstatement) [172-174], a drug-related stimulus (cue-induced reinstatement) [175, 176], stress (stress-induced reinstatement) [177] or by the context (context-induced reinstatement) [178].

The neurobiological substrates underlying reinstatement of instrumental responding for cocaine and heroin have been shown to involve the dopaminergic system and a broad neural network that includes the BLA, AcbC, prefrontal cortex (PFC) and particularly the glutamatergic projections from PFC to AcbC [179-183].

In the abstinence-relapse procedure, rats are trained to self-administer a drug for a short period of time, and are subsequently subjected to a forced-abstinence (maintained in their home cages) [168] or a self-abstinence wherein rats are trained to self-administer the drug under punishment, i.e. electric foot shocks [169-171]. Rats are then re-exposed to self-administration and often display a progressive increase in responding that reflects their propensity to relapse.

At the neurobiological level, forced-abstinence relapse has been shown to be dependent upon the dorsolateral striatum (DLS) [168, 184], suggesting a distinction in the psychological mechanisms subserving reinstatement following extinction and relapse following abstinence. Although the neurobiological basis of the relapse following self-abstinence remains to be more precisely described, projections from the Acb Shell to the lateral hypothalamus have been shown to be involve in relapse for alcohol [170].

b. *Loss of control over drug intake*

Ahmed and Koob developed the first model characterising the loss of control over cocaine [185] and heroin [186] intake, thereby challenging the 40 years old notion that rats “titrate” their intake to maintain an optimal level of reinforcement for as long as they have access to the drug. In these studies, rats are trained to self-administer drugs under short access conditions (ShA, one hour per day) under FR-1 schedule of reinforcement for several sessions. Then a group of rats is

exposed to long access (LgA, FR-1, 6 hours for cocaine and 11 hours for heroin) while another group of rats is maintained under ShA. LgA triggers a rapid increase in drug intake also known as escalation as compared to ShA. Not only LgA rats escalate their drug intake over the 6 or 11 hours but they also display higher drug intake during the first hour as compared to ShA rats. This escalation in intake is associated with intracranial self-stimulation thresholds [187], suggesting a shift in the hedonic set-point of LgA rats as suggested in the hedonic-allostasis theory of addiction [64, 66, 111, 188]. It is important to note that not all the rats escalate their drug intake. Indeed, McNamara and colleagues showed that only a subpopulation of rats escalated heroin self-administration when given LgA [189], however, inter-individual differences in escalation have been under-investigated.

Interestingly, at the neurobiological level, escalation of heroin intake has been shown to depend upon the kappa opioid receptors and the corticotropin-releasing factor R1 in the extended amygdala [190, 191].

c. *Multi-dimensional model of addiction*

Considering the limitations of the models discussed above, which did not encapsulate key aspects of addiction, such as inter-individual differences and compulsivity, Belin and colleagues [174, 192, 193] developed a preclinical model of cocaine addiction factoring in several behavioural features reminiscent of the diagnostic criteria of the disorder as defined in the DSM [139], i.e. the "3-criteria" model. In this model, rats are trained to self-administer cocaine under a fixed ratio 5 schedule of reinforcement during 3.5-hour daily sessions that comprise 2 periods during which the drug is not available and signalled as such. After 85 days of self-administration, rats are challenged for their motivation under a progressive ratio schedule of reinforcement, their ability to refrain from seeking the drug and their propensity to maintain self-administrating despite punishment. Only 20% of the population displays these 3 criteria that also predict increased propensity to escalate and vulnerability to relapse.

That model has been used to identify that high impulsivity trait, as characterised in the five choice serial reaction time task [194], predict the development of compulsivity, as revealed by the persistence of cocaine self-administration despite punishment. At the neurobiological level,

3-criteria rats have been shown to display a persistent impairment in synaptic plasticity in the ventral striatum and prefrontal cortex [195, 196]. Interestingly this altered synaptic plasticity in prefrontal cortex associated here with compulsive drug taking behaviour has been also shown to be associated with compulsive drug seeking behaviour using the seeking-taking task (see below). This is in marked contrast with the data obtained from sensitisation or short access drug self-administration which have consistently identified that drugs facilitate synaptic plasticity within the corticostriatal circuit.

d. Seeking-taking task and compulsivity

Drug addicts display the compulsive nature of their behaviour when they are engaged in foraging for their drug, not necessarily when they take their drugs. Seeking (preparatory) and taking (consummatory) responses are psychologically and neurobiologically dissociable, but procedures such as the "3-criteria" model, do not offer such dissociation. Therefore, it is important, spatially and temporally to dissociate them in behavioural tasks aiming to operationalise the compulsive nature of drug seeking. The seeking-taking task, developed in Everitt's lab, relies on a two-link heterogeneous chained schedule of reinforcement to dissociate the seeking from the taking responses. This model will be more described in Chapter 3 but briefly: rats are trained to respond on a seeking lever that is never paired with the drug but responding on which gives the opportunity to access a taking lever, a response on which results in drug delivery. Pelloux and colleagues elegantly introduced a probabilistic punishment schedule wherein 50% of seeking responses result in punishment (mild foot-shock) without access to the taking lever. Using this model, subpopulations of rats were shown to maintain their seeking responses despite adverse consequences, thus were characterised as compulsive [197-200]. This model has been used to demonstrate that compulsive cocaine seeking behaviour is associated with a hypoactivity of the PFC neurons, which optogenetic stimulation restore sensitivity to punishment [198].

This model is very relevant for probing the compulsive component of cocaine or alcohol seeking behaviour, however it fails to capture the stimulus-bound aspect of drug seeking and taking behaviour displayed by drug addicts. Moreover, it does not allow the measurement of

drug seeking responses in a drug-free state. Thus, considering the analgesic properties of heroin that would be a confounding factor, this model is not suitable for the operationalisation of compulsive heroin seeking behaviour.

e. Second order schedule of reinforcement

Seeking drugs for protracted periods of time under the control of drug-paired cues has been operationalised using second order schedules of reinforcement [146, 201, 202]. These schedules of reinforcement will be the focus of chapter 3. Briefly, rats are trained for a protracted period of time to seek the drug under FI schedules of reinforcement, often over 15 min periods, but their responses are reinforced by the contingent presentation of drug-paired cues, acting as conditioned reinforcers.

Overtraining under this schedule of reinforcement has been suggested to trigger the development of incentive habits. That procedure has helped elucidating the psychological and neural mechanisms associated with the development of cue-controlled drug seeking habits such as the elucidation of a functional shift from the ventral striatum (AcbC) to the dopaminergic-dependent DLS control over behaviour when it becomes a habit.

4. Limitations of these models

None of these models offers construct validity enough with regard to the multi-facetted nature of addiction. However, a selective combination of these approaches may help developing a procedure that enables the identification of individual vulnerability to develop compulsive drug seeking habits in rats trained to seek heroin over prolonged periods of time in a drug-free state bt under the controlled of drug-paired conditioned stimuli acting as conditioned reinforcers.

Objectives

The overarching aim of this PhD research project was to characterise the vulnerability to develop compulsive heroin seeking habits and identify their psychological, behavioural, neural and cellular basis.

While preclinical models of addiction have successfully operationalised compulsive cocaine

taking or seeking behaviour (respectively by the three criteria model and the punished seeking-taking task), a preclinical procedure enabling the identification of inter-individual differences in compulsive heroin seeking behaviour is yet to be developed. This is likely due to the difficulty of measuring persistence of heroin self-administration despite punishment as heroin has analgesic properties which lower the aversiveness of electric foot-shocks .

In order to circumvent this confounding factor, I have designed a new procedure of compulsive heroin which crystalises behavioural hallmarks of addiction, namely, the habitual, cue-controlled and compulsive nature of heroin seeking behaviour displayed by individuals suffering from an addiction to heroin. This model (extensively described in Chapter 3) capitalises on the second order schedule of reinforcement, which promotes the emergence of incentive habits, and utilises contingent punishment during drug-seeking 15-min intervals, especialy the first drug-free seeking period, to identify individual vulnerability to develop compulsive heroin habits.

Following the development and validation of this new procedure, I tested the hypothesis that the behavioural manifestations of punishing drug seeking over prolonged periods of time in a drug-free state observed in the heroin model were generalisable to cocaine. Thus, I carried out a similar experiment with cocaine as a reinforcer, and parametrically compared compulsive drug seeking behaviour between rats exposed to either heroin or cocaine.

Having validated this new procedure, I endeavoured to identify behavioural markers of vulnerability to develop compulsive heroin seeking habits. As previously described (and additionally reviewed in Chapter 4), individuals suffering from an addiction to cocaine display overlapping behavioural or personality traits with those suffering from an addiction to heroin. However, heroin and cocaine addicts dramatically differ in many behavioural and personality dimensions, including anxiety, decision making and impulsivity, thereby suggesting that behavioural traits of vulnerability that have been identified for cocaine addiction may not be relevant for heroin addiction. In preclinical research, while various research groups have extensively assessed the behavioural endophenotypes of vulnerability to cocaine addiction, very few studies have been investigated those involved in the vulnerability to develop heroin addiction (see Chapter 4). Therefore, I carried out longitudinal studies in which I first characterised several behavioural traits in a drug-naïve state, namely, anxiety, decision making,

locomotor reactivity to novelty, novelty-induced place preference, attribution of incentive salience to a stimulus and sensitivity to natural reward which have all been associated with a stage of the development of cocaine addiction Then, rats were subjected to heroin self-administration under the newly developed procedure to assess the inter-individual vulnerability to develop habitual and compulsive heroin seeking behaviour.

The development of this novel model of heroin addiction also enabled the investigation of the neurobiological substrates of compulsive drug seeking behaviour and the identification of the neural and cellular basis of incentive habits. As previously mentioned (and additionally reviewed in Chapter 5), neurobiological substrates of compulsive drug seeking have been identified over the past decade, but they pertain exclusively to cocaine addiction. In addition, neurobiological basis of incentive habits remains to be elucidated. Therefore, I investigated the neural and cellular correlates of heroin (and cocaine) seeking habits, and compulsivity from rats trained in the new procedure. Deploying an array of molecular biology techniques mostly relying on the combination of *in situ* hybridization-based hotspot analysis and quantitative polymerase chain reaction (PCR), I assessed the differential pattern of expression of molecular markers of cellular plasticity and candidate genes within several structures of the corticostriatal circuitry in rats displaying incentive habits for cocaine or heroin as well as compulsive drug seeking behaviour.

Analyses of the gene expression of striatal molecular markers revealed adaptations not only in neurons but also within astrocytes. This cell-type, primarily known to maintained extracellular homeostasis and to provide energetic supply to neurons, is increasingly considered to play an important role in various psychiatric disorders, including drug addiction (as described in Chapter 6). Therefore, in order further to characterize the contribution of astrocytes to the drug-induced adaptations in the striatal markers udner investigation I measured striatal DAT protein levels both from frozen tissue (containing neurons + astrocytes) and from primary astrocytes culture from rats trained to self-administer cocaine or heroin under different schedules of reinforcement. This offered new insights in the potential contribution of astrocytes in the functional shift from the ventral to the dorsolateral striatum subserving the transition from goal-directed to habitual drug seeking behaviour observed in the emergence of incentive habits.

CHAPTER 2: GENERAL MATERIALS AND METHODS

Behavioural experiments

1. Subjects

Male Sprague Dawley rats (*Charles River laboratories, Kent, United Kingdom*) weighing approximately 300g upon arrival were housed 4 per cage for a week of habituation (ad libitum access to food and water) and maintained under a reversed 12-hour light/dark cycle (light ON between 7.00pm and 7.00am). Following the habituation week, rats were singly housed and food restricted (15-20g of standard chow pellets daily) to maintain their body weight between 85 and 95% of their expected free-feeding body weight. Rats were then subjected to various procedures conducted 6-7 days/week, in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986 amendment regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB) under the project licence number 70/8072.

2. Characterisation of behavioural traits

Each population of rats (n = 23/24) was screened for specific behavioural traits in different relevant tasks within longitudinal studies in order to characterise potential behavioural factors of vulnerability to develop some features of heroin addiction.

Anxiety-related behaviour, novelty-induced place preference and locomotor reactivity to novelty were measured using a video tracking system on an Elevated Plus Maze (EPM), four Conditioned Place Preference (CPP) boxes and four Open Fields (OP) respectively. Individual differences in incentive salience attribution to conditioned stimuli [203] and decision making were assessed in operant chambers in the AutoShaping task and the Rat Gambling Task (RGT) [204]. Sensitivity to natural rewards was conducted un a two-bottle choice procedure opposing water to saccharin.

a. Video tracking behavioural procedures

General description of the video tracking system

The hardware components of the system required to monitor the behaviour of rats in open spaces by video tracking (including the mazes) were purchased from *ViewPoint Behavior Technology®* (*Lyon, France*). The behavioural testing sessions were conducted in a room exclusively dedicated to this purpose. The different mazes (made of Plexiglas translucent to infrared light) were installed on a Plexiglas floor (120 x 120 cm) backlit by infrared light-emitting diodes and the rats' behaviour was recorded by contrast by two cameras equipped with infrared filters located on the ceiling directly above the floor. Two types of behavioural events were detected; the ambulatory motions, namely trajectory and speed of the animal, which were set up on the animal's centre of gravity, and the so-called "small movements" (grooming, stereotypies…) which were centred on the animal's muzzle. Acquisition and recording of behavioural events were controlled by VIDEOTRACK.v3 software (*ViewPoint Behavior Technology®, Lyon, France).* All the mazes were cleaned between each session with 20% ethanol to prevent any potential behavioural bias triggered by odours left by the previous animal.

Anxiety-related behaviour

The EPM has been intensively used to assess anxiety-related behaviours [205-207] and validate the therapeutic efficacy of anxiolytic drugs [208]. As stipulated by its name, the EPM is 50 cm elevated above the floor and is composed of two open arms, two closed arms and a central platform (**Fig. 2.1**). The test stems from rats' natural fear of open and brightly lit spaces. Therefore, the luminosity was measured in each portion of the EPM and the light adjusted to reach an intensity of 40 Lux in the central platform, 50 Lux in the open arms and 30 Lux in the closed arms which confer a protected-like compartment for rats [209]. The tests were conducted during the dark phase of the dark/light cycle.

At the beginning of a test session, the rat was placed on the central platform with its head pointing towards an open arm (always the same arm for all subjects). The rat was then allowed to explore the maze for 5 minutes while its movements were monitored. The time spent and the number of entries into each of the arms were recorded as well as discrete exploratory

movements such as scanning and head dipping at the different areas of the open arms, these exploratory events reflecting low anxiety level (**Fig. 2.1**). An anxiety score was calculated for each subject as a percentage of time spend in the open arms over the time spend in all the arms. Individuals belonging to the upper and lower quartiles (25%) of each population were considered Low Anxious (LA) and High anxious (HA), respectively [209, 210] (**Fig. 2.3-C**).

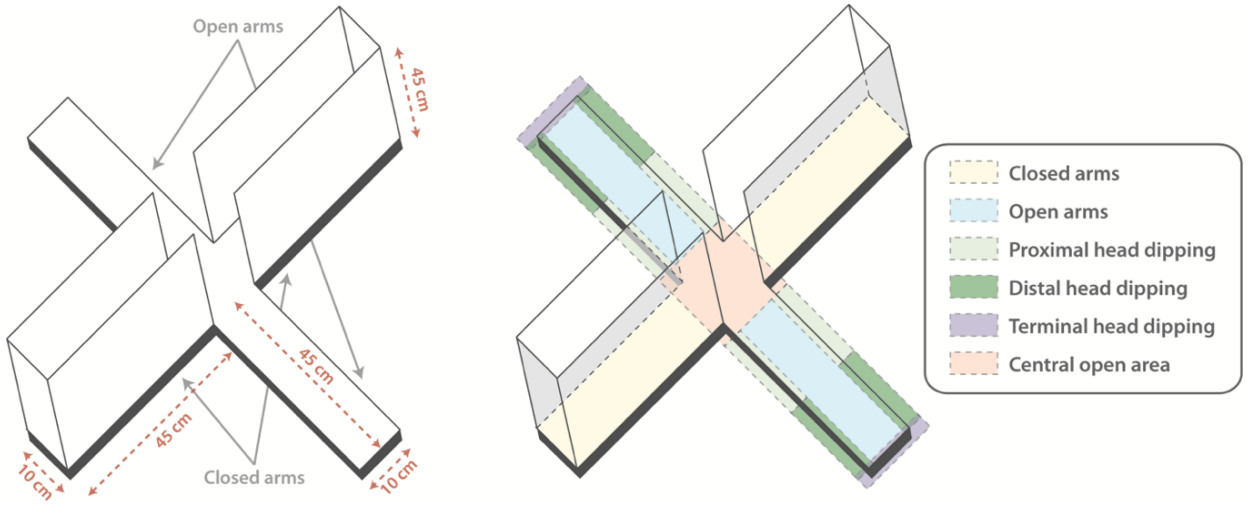


Figure 2.1: Elevated Plus Maze (EPM). *The EPM is 50 cm elevated above the floor and is composed of a central platform (10x10 cm), 2 open arms (45x10 cm) facing each others and 2 closed arms (45x10x45) also facing each others. The behaviour of each rat is recorded in the different designated areas: closed arms, open arms, central area for the general body trajectories, and in the different territories of the open arms (proximal, distal and terminal) for the detection of fine exporatoty behaviours (i.e. head dipping).*

Novelty-induced place preference

The novelty-induced place preference (NPP) test adapted from Damaudéry et al. [211] was performed in four conditioned place preference boxes which are composed of one narrow central corridor surrounded by two large compartments as previously described [212] (**Fig. 2.2-A**). Two guillotine doors are located at both extremities of the central corridor giving access to the other two compartments which are closed by Plexiglas lids to decrease the luminosity inside and to protect rats from any external stimuli. The configuration of the boxes is adjustable such as the walls and floors can either be black or white and their texture smooth or rough (**Fig. 2.2-B**). The different configurations were randomized between individuals. The luminosity was measured in each part of the box and the light adjusted to reach an intensity of 0-0.2 Lux in the two large compartments and 10 Lux in the central corridor. The tests were conducted during the dark phase of the dark/light cycle (7.00am-7.00pm).

The single test session for each rat was composed of three successive steps as previously described [212]. The animal was first placed in the central corridor with the two doors closed for a short habituation period of 5 min. Then the rat was placed in one of the two compartments, considered as the "familiar compartment" (randomized between individuals) for 25 min. In the final step, the animal was placed in the central corridor 5 seconds before the two doors were opened giving free access to all the compartments of the box (**Fig. 2.2-C**).

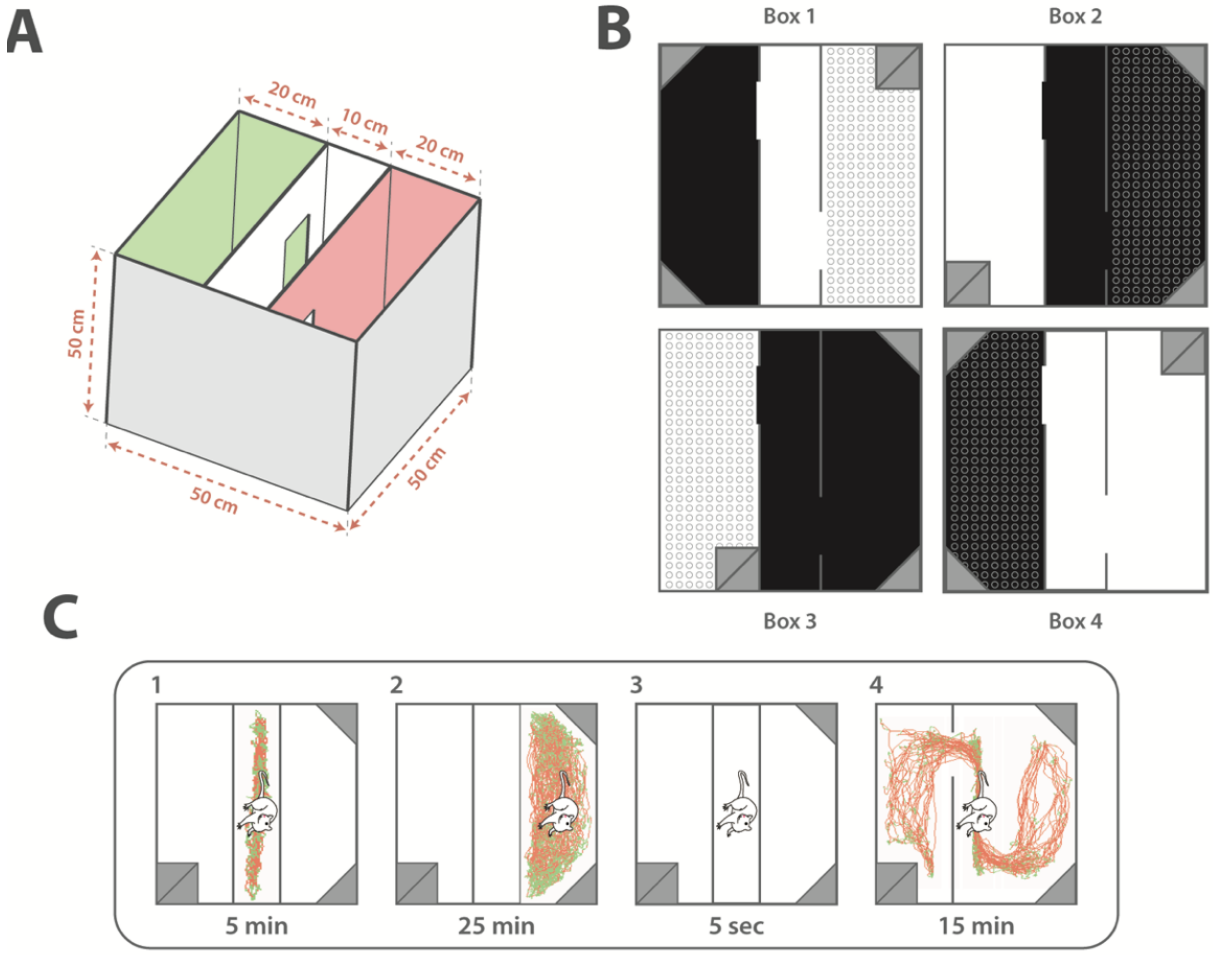


Figure 2.2: Novelty-induced place preference test. ***A)*** *Each CPP box (50x50x50 cm) is composed of two compartments (20x50x50 cm) and a central corridor (10x50x50 cm). The compartments are communicating with the corridor by 2 guillotine doors.* ***B)*** *The colours and textures of the floors and walls can be changed to be either white or black and smooth or rough. Removable prisms can be added to change the overall shape of the compartments without altering their size.* ***C)*** *Rats were initially habituated to the central corridor for 5 min (1), then to the familiar compartment for 25 min (2). Rats were then placed in the central corridor for 5 seconds (3) before opening the doors, giving them access to the entire box that they explored for 15 min (4).*

The time spent in each of the 2 compartments (familiar and novel) was recorded and a score of NPP was calculated for each subject as a percentage of time spend in the novel compartment over the time spend in the two compartments. Individuals belonging to the upper and lower

quartiles (25%) of each population were considered as High Novelty Preference rats (HNP) and Low Novelty Preference rats (LNP) respectively (**Fig. 2.3-B**).

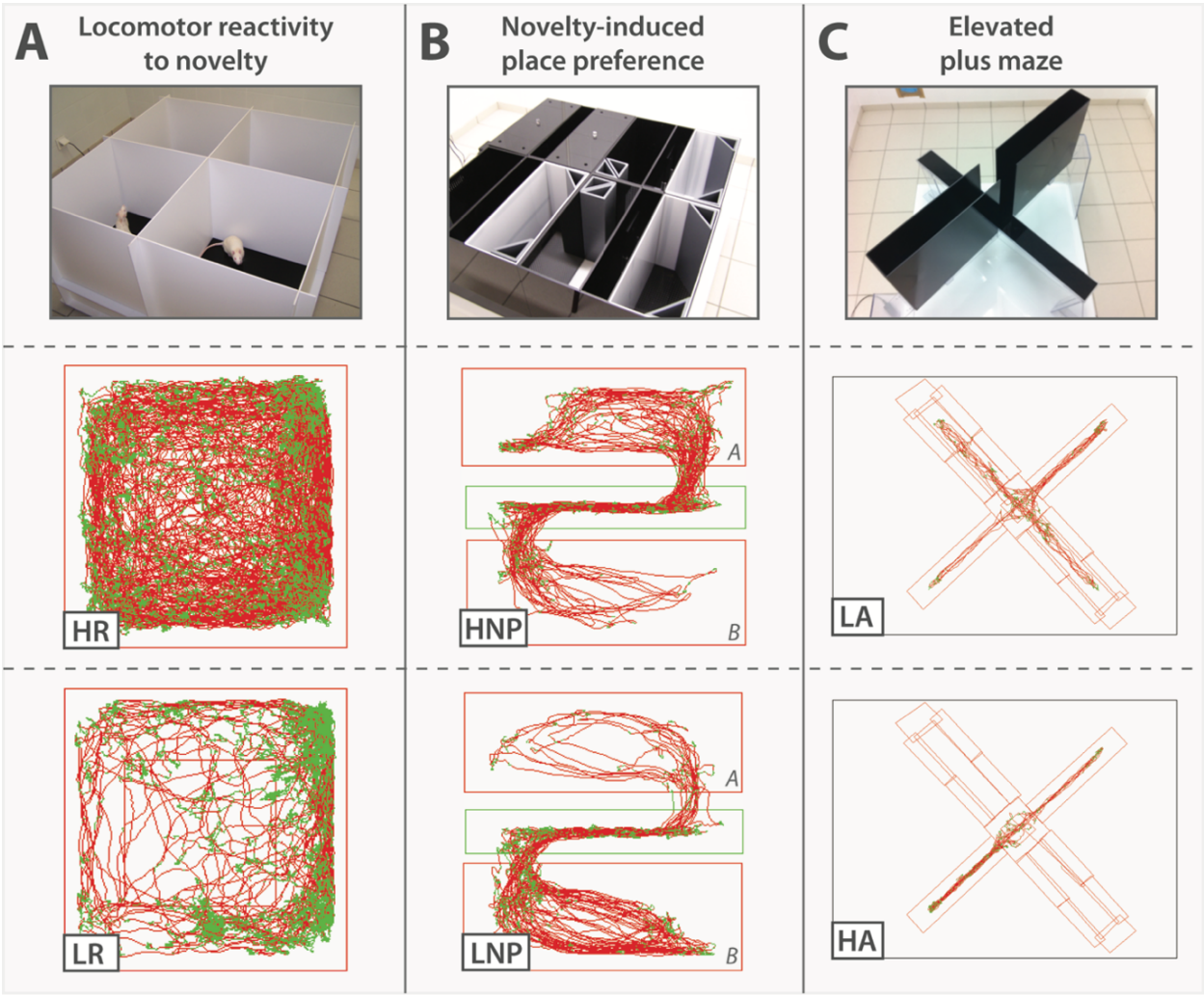


Figure 2.3: Video tracking-based behavioural tests. ***A)*** *Picture of 4 open fields wherein rats are tested for the locomotor reactivity to novelty. Below are illustrations of the tracks of representative HR and LR rats obtained from ViewPoint software (red: body motions; green: small movements). HR rats display a greater locomotor activity than LR rats.* ***B)*** *Picture of the 4 CPP boxes displaying the four different configurations used to assess NPP. Below is the representation of the box and the 2 compartments (A: novel; B: familiar) and the movements recorded for representative HNP and LNP rats. HNP rats spend a greater amount of time in the novel compartment compared to LNP rats.* ***C)*** *Picture of the EPM used to assess anxiety-like behaviour. Below is the representation of the maze and the movements of a representative LA and HA rat in its different areas. LA rats spend a greater amount of time in the open arms compared to HA rats which stay most of their time in the closed arms.*

Locomotor reactivity to novelty

The locomotor reactivity to novelty test adapted from Dellu et al. [213] was conducted in four open fields (50 x 50 x 50 cm) during the light phase of the dark/light cycle (7.00pm-7.00am) under a light intensity similar to those present in the holding rooms (around 550 Lux at the centre of

the open field). Rats were placed in the open fields for two hours and their locomotor activity was recorded throughout. Individuals whose total distance was included in the upper and lower quartiles (25%) of a population were considered High Responders (HR) and Low Responders (LR) rats, respectively (**Fig. 2.3-A**).

b. Operant conditioning procedures

Two days prior to any task based on the deliverance or self-administration of natural rewards (i.e. 45 mg food pellets, *TestDiet, USA*), rats received 20 pellets in their home cage to avoid any potential food neophobia. The first day of training for all rats was a single session of "magazine training" to learn that, and where, food pellets were delivered in the experimental chambers. This session consisted of 60 pellets delivered according to a 30s-variable interval (VI) schedule.

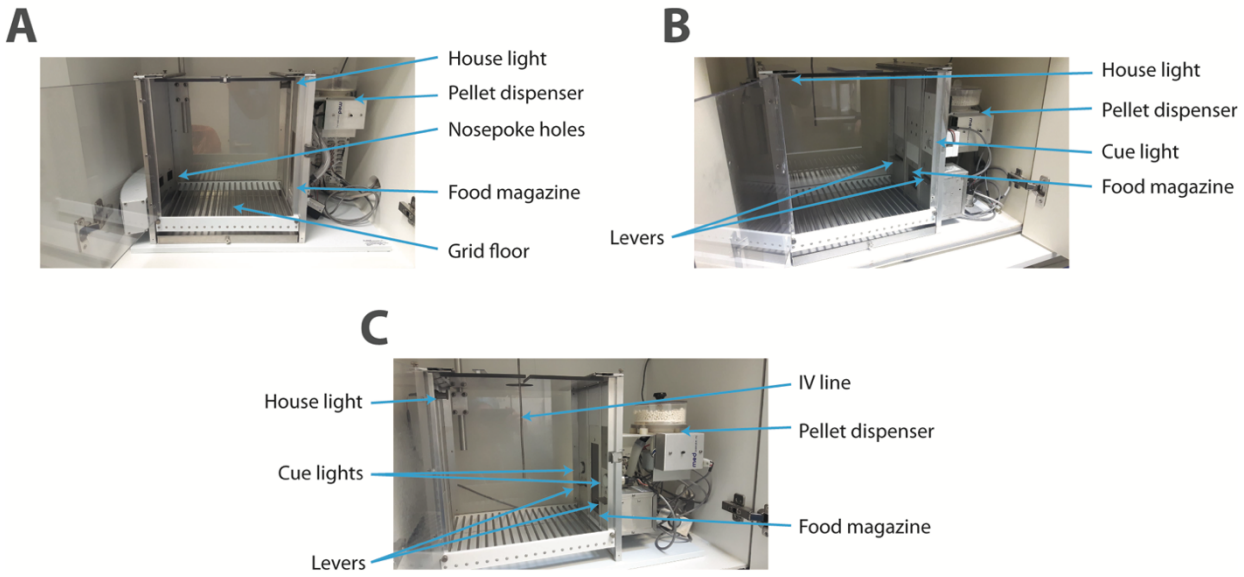


Figure 2.4: Operant chambers used for RGT, Autoshaping task and self-administration procedures. A) *5-holes operant chambers: Each of the 8 chambers was illuminated by a house light located opposite a curved wall containing five nosepoke appertures. A pellet dispenser supplied pellets to a food magazine facing the curved wall.* ***B)*** *Autoshaping chambers: Each of the 12 boxes was illuminated by a house light. Chambers also contained two retractable levers (4 cm wide), located either side of a food magazine, to which pellets were dispensed via an external pellet dispenser. Cue lights (dispensing a mixed light: green, red and yellow) were located above one of the two levers.* ***C)*** *Self-administration chambers: the 24 self-administration boxes had the same configuration the chambers described above, with slight modifications. Namely a stimulus light (2.5W, 24V) was located above each lever and a flexible tube (IV line), protected by a metal spring set up on a pivoting arm, was linked to a perfusion pump at one end and to the rat catheter on the other side..*

Apparatus

Three different configurations of operant chambers were used to perform the autoshaping task, the rat gambling task and the self-administration procedure (**Fig. 2.4**). Each chamber (31.8

cm x 25.4 cm x 26.7 cm) (*Med associates, St. Albans, USA*) was located within a ventilated sound-attenuating cubicle. Front and back panels of the test chambers were aluminium, while right and left walls and the roof were transparent acrylic plastic with stainless grid floor. A pellet dispenser was installed behind the front wall of each chamber, supplying food pellets to a food magazine located 2 cm above the grid floor. Each test chamber was illuminated by one 3-watt light bulb (house light) during the experimental session. The scheduling and recording of experimental events were controlled by either MED-PC IV software (*Med Associates, St. Albans, USA*) or Whisker software suite (*Whisker, Cambridge, UK*). Each experimental session was carried-out in the same operating chamber for each subject.

Rat gambling task

The experimental sessions were conducted in the five-holes operant chambers (**Fig. 2.4-A**). The procedure was structured around eight consecutive training sessions followed by a test session (**Fig. 2.5**). Rats initially learnt to poke in each of the four illuminated nose poke holes located within the curved wall opposite the magazine to obtain one pellet delivered to the magazine. Nose pokes in the middle inoperative hole were recorded, but had no consequence. Sessions continued until rats obtained 100 pellets within a session (30-min cut-off). After two of these free choice training sessions, rats were trained under a forced choice procedure whereby each hole was active for 7 min 30 seconds (25 pellets cut-off), according to a random presentation within the four forced choice sessions (**Table 2.1**).

Session	Active holes			
1	1	2	4	5
2	2	5	1	4
3	4	1	5	2
4	5	4	2	1

Table 2.1:sequences of single hole activation over the four sessions of the forced choice procedure

These sessions were implemented to avoid biases towards a particular side or hole. Rats were then trained in two consecutive free-choice sessions during which each nose poke in one of the four active holes results in the delivery of one pellet (first session) and two pellets (second session) for the animals to learn that responding can result in the opportunity to receive 1 or 2

pellets.

Finally, rats were tested on the RGT during which the magnitude of reward and punishment varied between the four holes. Poking in two of the four holes delivered one pellet immediately, but with a 0.25 (hole **1**) or 0.5 (hole **4**) probability of a concomitant time-out (12s or 6s respectively). Poking in the other two holes resulted in the delivery of 2 pellets immediately, but with a 0.5 (hole **2**) or 0.25 (hole **5**) probability of a longer time-out (222s or 444s respectively). Advantageous choices were those associated with responses made to holes **1** and **4** because they enabled rats to complete more trials and obtain more pellets within the one hour-single session. Animals varied in the extent to which they maximised reward on this task, as measured by the percentage choice of the advantageous options. Rats belonging to the upper and lower quartiles of the population were characterised as good (GDM) or bad decision maker (BDM), respectively.

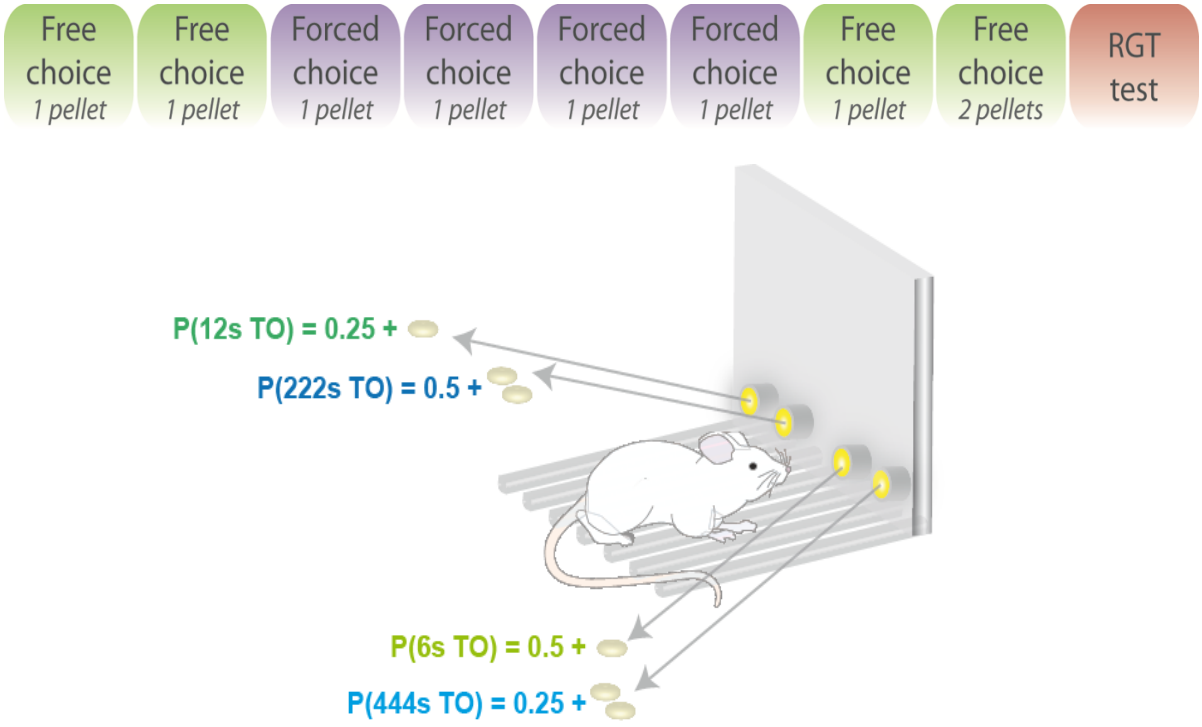


Figure 2.5: Experimental design of the rat gambling task. Top panel: *timeline of the experiment. Rats were trained for eight consecutive days alterning free and forced choice sessions. Decision making was assessed during the final RGT session.* ***Bottom panel:*** *illustration of the outcome of the choices during the single RGT session.*

Autoshaping task

The experimental sessions were conducted in the autoshaping-configured operant chambers (**Fig. 2.4-B**) for 5 consecutive daily sessions. During training rats learnt to associate the

presentation of a compound stimulus composed of an illuminated lever (Cs) and lever presentation with the delivery of one pellet in the absence of any instrumental contingency.

On each trial, both retractable levers were inserted in the chamber and the Cs was illuminated above one of them (called active) for 8 seconds according to a VI schedule centred on 90 seconds (VI-90s). After 8 seconds, the Cs was turned off, the active lever was retracted (the inactive lever stayed inserted in the chamber the entire session) and one pellet was delivered in the food magazine. Once the pellet was delivered, another VI-90s started again and this cycle continued until the end of the one-hour session. During the interval of 8 seconds, active lever presses were recorded as sign-tracking (autoshaping) events while head entries into the food magazine were recorded as goal-tracking events. Inactive lever presses (uncoupled with the Cs) were also recorded as an indicator of general activity. Individuals within the upper and lower quartiles of the population stratified on their level of sign tracking (lever presses) over the last three sessions, were considered sign-trackers (ST) and goal trackers (GT), respectively.

c. *Saccharine preference*

Prior to testing, rats were water deprived for 2 hours in order to avoid any potential bias of performance by individual differences in thirst. Rats were weighed prior to the beginning of each session in order to be able to compute a fluid intake/body weight ratio. The two-bottle choice sessions were conducted daily, in cages similar to the home cages but with no bedding so as to ensure the environment was different to the home cage. During the initial four habituation sessions, rats were given access to two bottles of water which were weighed before and after the session to measure general fluid intake. Following these four sessions, two test sessions (two hours each) were conducted wherein rats had access to one bottle containing water and one bottle containing 0.2% saccharin (*Sigma*). Bottles were weighed just before and after the session to measure the intake of both water and saccharine solution. One hour following the beginning of each test session, bottles were swapped in the cage for the remaining 1 hour period to control for the development of a side preference bias. The ratio between saccharine intake over the total fluid intake was calculated, and individuals belonging to the upper and lower quartile of the population stratified on saccharine preference over the two sessions were considered as High saccharin Preference (HP) and Low saccharin Preference (LP) rats, respectively [214].

3. Surgeries and drugs

Rats that underwent drug self-administration procedures received antibiotic treatment starting one day prior to surgical procedure and continued for 5 consecutive days (*oral Baytril 2.5%, Bayer, 2.5ml/kg diluted daily in water*). They were anesthetised by intramuscular injection of a ketamine/Xylazine mixture (*Ketalar, 90 mg/kg, i.m, Bury St. Edmunds, UK; Rompun, 6.7 mg/kg, i.m., Bury St. Edmunds, UK*) and a silastic catheter (*CamCaths, Cambridge, UK*) was implanted into their right jugular vein as previously described [215]. Catheters were topped by a steel cannula surrounded by a nylon mesh that was subcutaneously sutured between the scapulae (**Fig. 2.6**). Following recovery, catheters were flushed daily by injection of 50 to 100 µl of sterile physiological saline (0.9% sodium chloride) supplemented with heparin (20 IU/ml) to maintain patency.

Heroin and cocaine hydrochloride (*McFarlan-Smith, UK*) were dissolved in sterile physiological saline at a final concentration of 0.04mg/0.1mL and 0.25mg/0.1mL respectively, and stored at 4°C.

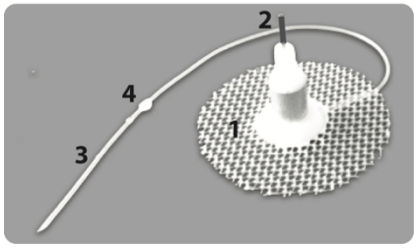


Figure 2.6: Intravenous catheter. *Catheters are made of a silastic tube (3) linked to a steel cannula (2). The bottom of the cannula is surrounded by a nylon mesh (1) which is subcutaneously sutured between scapulae. The tube extremity (3) is inserted in the jugular vein until the silicon bubble (4). The rest of the tubing goes subcutaneously from the jugular vein to the scapulae.*

4. Self-administration procedures

The different procedures consisted of consecutive daily sessions of drug self-administration, each session was carried out in operant chambers (**Fig. 2.4-C**). For each experiment, rats had access to both an active and an inactive lever, the location of which was randomised between sessions and individuals. In this study, 3 types of reinforcers were used independently, namely cocaine, heroin and food pellets.

a. Fixed-Ratio (FR) schedule of reinforcement

In each experiment, rats were initially trained under a FR-1 schedule of reinforcement until they acquired instrumental responding (significantly higher level of responding on the active lever than on the inactive lever thereby showing discrimination and stable rate of injections).

Each active lever press resulted in the immediate delivery of a reinforcer (heroin: 0.04mg/0.1ml/5.7s/infusion, cocaine: 0.25mg/0.1 ml/5.7s/infusion, one 45mg food pellet) and a 20s presentation of a stimulus light above the active lever (that will become the conditioned stimulus (Cs) through Pavlovian conditioning). During these 20 seconds (time-out period) the house light was turned off and the levers were retracted. At the end of the time-out, the house light was turned back on, the CS switched off and the two levers inserted back into the chamber. The number of drug infusions was limited to 30 and the number of natural reinforcers to 100 pellets during each 2-hour daily session. Inactive lever presses had no programmed consequences but were recorded to monitor the specificity of the instrumental response.

b. Fixed-Interval (FI) schedules of reinforcement

The FI schedules of reinforcement enable the quantification of seeking behaviour, e.g. instrumental responding in the absence of the unconditioned stimulus. In the following experiments, FI protocols were used progressively up to 15 minutes, a corner stone of the second order schedule or reinforcement (SOR) [216] which has been extensively used to probe the psychological and neurobiological substrates of incentive habits [145]. During the first FI session, seeking periods were introduced wherein rats could only receive drug infusion by pressing the active lever once a 1-min interval had elapsed (called FI-1). The FI was subsequently increased every day from FI-1 to FI-2, FI-4, FI-8, FI-10, up to FI-15. Rats were subjected to three sessions of FI-15 wherein drug infusion was available by pressing the active lever once a 15-min interval had elapsed (used as baseline sessions prior to the introduction of SOR). During these sessions, the number of infusions was limited to 5 (2 hours cut off).

c. Second Order schedule of Reinforcement (SOR)

In a FI-15(FR-10:S) second order schedule of reinforcement drug seeking, measured over prolonged periods of time (15 min) in a “drug-free” state is invigorated by the contingent presentation of a drug-paired CS (1s) every tenth active lever press. These CSs act as conditioned reinforcers, bridging delays to reinforcement and eventually facilitating the instantiation of incentive habits [146]. The number of infusions (which were available once the animal pressed ten time on the active lever after each 15-min interval has elapsed), was limited to 5 during each

session.

5. Tissue collection

Rats were deeply anaesthetised by Isoflurane inhalation and decapitated. Brains were harvested in less than 30 seconds in order to prevent any cellular/protein degradation. Brains intended for in situ hybridization, western blot, and quantitative polymerase chain reaction (qPCR) assays were flash-frozen by immersion in -35°C isopentane for 5 min then placed in dry ice and stored at -80°C.

In situ hybridisation required to process the whole brain into 12 µm-thick coronal sections using a cryostat (*Leica CM3050S*). Serial brain sections were collected on gelatine-coated slides and stored at -80°C until further use.

Western blot and qPCR assays required to micro-punch using a micro-puncher (1 mm diameter) selected brain structures from 300 µm-thick coronal sections performed using a cryostat.

Samples dedicated to qPCR assay and *in situ* hybridisation had to be preserved from RNA degradation. Thus, every piece of equipment and consumables in contact with the brains or the samples were either sterile (autoclaved where possible) or sprayed with a RNase decontamination solution (*RNaseZAP, Ambion*).

Brains from which astrocytes were cultured were positioned on an acrylic frame (*Plastics one inc.*) on ice and processed into 2 mm-thick coronal sections. The same structures as those used for micro-punch and subsequent assessment of protein expression by western-blot were dissected on ice and immersed in 150 µl DMEM+ (*DMEM High Glucose, supplemented by 10% FBS, 1% penicillin/streptomycin and 1% Glutamine, Gibco*) in order to preserve cellular integrity required for the subsequent steps of cell culture.

Molecular Biology experiments

1. Astrocytes primary cell culture

Samples from freshly dissected brains were mechanically dissociated with a pellet mixer (*Argos Technologies*) in 300 µl of DMEM+ and subsequently vortexed then centrifuged at 1300 rpm during 5 minutes. Supernatants were removed and the pellets suspended in 300µl of fresh DMEM+. Samples were vortexed and centrifuged once again, supernatants were removed and cells were suspended in 1mL DMEM+. These steps were necessary to wash the cells and discard molecular compounds such as apoptotic factors which could jeopardise the viability of the cultured cells. Following 10 minutes of decantation, supernatants were plated directly into 12-12-well plates and stored in an incubator (*Binder, Germany*) with a controlled environment set up at 37°C, 5% CO_2. The decantation step was necessary to discard the heavy weight contaminants (i.e. pieces of tissue or fibres) while cells stayed in suspension.

Four days later, 500µl of medium were removed and replaced by 500µl of fresh medium supplemented with basic Fibroblast Growth Factor (*bFGF, Gibco*) at a final concentration of 10 ng/mL, which promotes astrocytes proliferation. Subsequently, over a period of 3 to 5 weeks, the whole medium was changed and the cells were treated with bFGF (10 ng/mL) weekly.

2. Western blot

Potential differences in protein levels between experimental groups were assessed by western blot from both cultured astrocytes and samples of structures punched from frozen brains.

a. Sample lysis

Following four to six weeks of culture, wells were emptied and cells received 100µl lysis buffer (*complete Lysis-M kit*, Roche) whereas punched samples were weighed and mixed with lysis buffer (10µl /mg of sample). The following steps of lysis were conducted on ice to prevent the activation of anti-proteases. Following pipette homogenisation and centrifugation (15 minutes, 15000g, 4°C), supernatants were collected and protein levels were quantified using the fluorometric Qubit (*Invitrogen*) assay or a spectrophotometer assay (*nanodrop, ND-1000*). Protein lysates were stored at -20°C until used for western blot assays.

Proteins at this stage remained folded in their quaternary structures maintained mainly by disulfide bonds and covalent interactions. To improve the electrophoresis migration and the accessibility of antibodies to their epitopes, 20 µg of proteins were mixed with two reagents. A reducing agent, namely Dithiothreitol (DTT, 0.5M) (*Sigma*) which disrupts the disulfide bonds and a buffer allowing for maximum activity of DTT namely Lithium Dodecyl Sulphate buffer (LDS buffer, 0.5X) (*LDS Sample Buffer 4X, NuPage, Novex, Invitrogen*). Covalent interactions were disrupted by heating up the mix between 95 and 100°C for 5 minutes. At this stage, proteins were linearised and ready for the electrophoresis migration.

b. Electrophoresis and transfer

Proteins were loaded in pre-casted polyacrylamide gels (*4-20% Tris-Glycine gels, Novex, Invitrogen*) secured in an electrophoresis system (*X-Cell SureLock Midi-cell, Invitrogen*) and proteins were separated for 1-1.5 hour at 220V. Transfer of proteins onto a nitrocellulose membrane (*iBlot Gel Transfer Stacks Nitrocellulose, Regular, Invitrogen*) was performed in a semi-dry transfer apparatus (*iBlot, Invitrogen*).

c. Antibodies incubation

Membranes were washed three times with a washing buffer (1X Tris Buffered Saline (TBS) / 0.05% Tween 20) to get rid of some chemical residues coming from the transfer step. To promote antibody-specific-binding, the non-specific sites were saturated by a two-hour incubation of the membranes in in a blocking buffer (1X TBS-0.05% tween-5% Bovine Serum Albumin (BSA)) at room temperature (RT). The primary antibody targeting the protein of interest was diluted at the desired concentration in the blocking buffer and incubated with the membranes at 4°C overnight. Membranes were washed three times with the washing buffer and incubated with the secondary antibody (targeting the host species of the primary antibody) diluted at the desired concentration in the blocking buffer for 1 hour at Room temperature (RT). Membranes were then washed three times with the washing buffer and the primary antibody targeting the loading control protein (host in a different species than the one used for the protein of interest) was diluted at the desired concentration in the blocking buffer and incubated with the membranes at RT for 1 hour. Membranes were washed three times with the washing buffer and incubated with the secondary

antibody (targeting the host species of the primary antibody targeting the loading control protein) diluted at the desired concentration in the blocking buffer for 1 hour at RT (both secondary antibodies were linked to a horse radish peroxidase, HRP). Membranes were finally washed three times with the washing buffer.

d. Signal detection

The western blot signal was captured using an electrochemiluminescence (ECL) detection system. The membranes were placed horizontally in the imaging system (*ChemiDoc-It, Ultra-violet products*) and received 1 mL of an HRP substrate (*Luminata, Millipore*). HRP (linked to the secondary antibodies) catalyses the oxidation of luminol when peroxide is present, leading to an emission of photons captured by a camera embedded in the imaging system.

The pictures taken by the camera were analysed with *ImageJ software* with which an optical density value for each band of each well corresponding to either the protein of interest or the control was attributed. The protein of interest / control protein ratio was calculated to exclude any experimental bias and used as dependent variable in subsequent statistical analyses.

3. Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) is a technique widely used to quantify the transcription products of genes, namely mRNA (messenger RiboNucleic Acid) reflecting the relative level of a specific mRNA between different conditions or experimental groups. The assessment of mRNA levels from punched samples (*see "tissue collection" section*) required initially to isolate the RNAs from genomic deoxyribonucleic acid (DNA) and other cellular contaminants. A complementary DNA (cDNA) bank from the mRNA extracted was then created and the target cDNA was amplified by a succession of enzymatic reactions requiring, as start points, the use of two short sequences of nucleic acid complementary to the target cDNA (called primers).

a. RNA extraction

RNA extraction was carried out by using the *Quick-RNA MicroPrep kit* (*Zymo Research*) in a RNase-free environment. RNA is very sensitive to degradation by RNases which are abundantly

present in a laboratory environment, thus, all the consumables and reagents used to carry-out qPCR were either sterile or sprayed with an RNase decontamination solution (*RNaseZAP, Ambion*). Manipulation of samples was carried-out under a laminar flow hood in order to prevent any contamination of the sample that could potentially lead to RNA degradation.

Punched samples were mixed with 300 µL of lysis buffer containing detergent to disrupt the tissue and chaotropic salts which destabilize proteins and stabilize nucleic acids. Following pipette homogenisation and centrifugation (1 minute, 16000g), supernatants were collected and mixed with 100% ethanol (300 µL), then transferred to a silica column. The association of chaotropic salts and ethanol creates an optimal environment for the exclusive binding of nucleic acid to the silica. The samples were centrifuged (30 seconds, 16000g) and washed with a washing buffer (400 µL) to discard the contaminants and stabilized only the nucleic acid within the column. Within the cells, nucleic acids are found in several forms such as genomic DNA, mitochondrial DNA, transfer RNA, small interfering RNA, messenger RNA and ribosomal RNA. To prevent any DNA contamination of the samples, the columns received a DNase treatment (5 µL of DNase1 diluted in 35 µL of digestion buffer) for 15 minutes at RT. The columns were centrifuged (30 seconds, 16000g) to discard the enzyme and its buffer. Columns were washed four times with a washing buffer (400 µL) and a centrifugation step (30 seconds, 16000g) was performed between each wash. Columns were then centrifuged (2 minutes, 16000g) to eliminate any liquid and the RNAs were eluted in 25 µL sterile water.

RNA levels were quantified using a spectrophotometer assay (*nanodrop, ND-1000*) and RNA extracts were aliquoted and stored at -80°C until the reverse transcription step.

b. Reverse transcription

Since PCR amplifies DNA sequences, RNA needs to be reverse-transcribed into cDNA in a reverse transcription reaction (RT) prior to the PCR. This reaction was performed by using the *RT*^{2} *First Strand kit* (*Qiagen*). Samples of RNA templates (between 25 ng and 5µg) were diluted in 10µL sterile water. They were then mixed with a reverse transcriptase solution containing the enzyme reverse transcriptase, some random oligomers, a mix of the four deoxyribonucleotides (*dATP: deoxyadenosine tri-phosphate, dCTP: deoxycytidine tri-phosphate, dGTP: deoxyguanosine tri-phosphate, dTTP: deoxythymidine tri-phosphate*) and a buffer providing the optimal

environment for the enzymatic activity (**Table 2.2**).

Component	Volume for 1 reaction
5X buffer BC3	4 µL
Control P2	1 µL
RE3 Reverse transcriptase mix	2 µL
Rnase free water	3 µL
RNA templates	10 µL

Table 2.2: Composition of the reverse transcription mix

The final mix was incubated at 42°C for 15 minutes (optimal temperature for the enzymatic activity) then the reaction was stopped by incubating at 95°C for 5 minutes. Sterile water (91 µL) was added to the reaction and the cDNA were stored at -80°C until the PCR step.

Random oligomers bind to their complementary RNA in a specific-sequence manner so that each RNA molecule will eventually be bound to at least one oligomer. At 42°C, the enzyme synthesises, from the oligomers, a complementary sequence of the RNA by adding dNTPs. At the end of the enzymatic reaction, a double strain containing the RNA template and the cDNA newly synthesised is obtained. Heating up the mix at 95°C allows the dissociation of the double strain structure and the cDNA can be used as a template for the PCR.

c. Polymerase chain reaction

cDNA templates of the target mRNA were specifically amplified and their quantity was estimated relatively to the fluorescence emission of an intercalant marker, namely SYBR Green. This reaction performed using the *RT² SYBR Green Mastermix* (*Qiagen*) requires five factors: cDNA sample, the enzyme (a DNA polymerase), a couple of primers (small sequences of nucleic acid) framing a sequence of the target cDNA (sense and antisense), the four deoxyribonucleotides and an alternation of temperature cycles. The reaction was structured around 40 consecutive cycles wherein temperature was alternated (see below) and was performed in the *CFX96 Real-Time PCR Detection system* (*Bio-Rad*).

In 96 non-skirted, low profile well-plates (*Eurogentec*), cDNA (1 µL) was mixed with *RT² SYBR Green Mastermix* (12.5 µL), *RT² qPCR Primer assay* (1µL) and sterile water (10.5 µL). The wells were sealed by optical flat cap stripes (*Eurogentec*), then briefly centrifuged and placed in the

PCR system.

The first cycle started with increasing the temperature to 95°C for 10 minutes which results in the activation of the DNA polymerase. Starting from the primer-cDNA double strands, this enzyme synthesises a sequence complementary to the target cDNA. The other cycles are a succession of maintaining the temperature to 95°C for 15 seconds and then at 60°C during 1 minute. At 60°C, the enzyme is inactivated, the newly synthetized strain hybridises with the template strain and a fluorescent intercalant agent (SYBR Green) binds to this double-strained molecule. In this phase, the fluorescence emitted by SYBR Green, which is relative to the quantity of double-strained molecules is recorded. The primers couple frames a little sequence of the target cDNA such as throughout the cycles only this sequence (called amplicon) will be amplified. The fluorescence, which is not quantifiable during the first cycles (due to the small quantity of double-strained molecules) increases proportionally with the quantity of newly synthesised amplicons (each cycle leads to the double amount of amplicon).

Since qPCR offers a relative quantification of the expression of target cDNA, each sample was always compared to a house keeping gene expression (from the same sample) which has to remain stable in the different experimental conditions. To verify the specificity of the amplification (i.e. to ensure that a single amplicon has been amplified) a melting curve was processed following the 40 cycles by increasing the temperature from 65°C to 95°C with a rate of 2°C per minute.

d. Data analysis

The data were collected and analysed by *CFX Manager Software* (*Bio-Rad*). Two types of graphs containing different information were obtained, namely the amplification curve and the melting curve (described in the next sub-section).

The amplification curve represents the level of fluorescence recorded at each cycle (**Fig. 2.7**). A threshold was adjusted to a value above the background and below the plateau phase, in the linear region of the amplification curve. A threshold cycle (Ct) for each sample was measured and corresponds to the cycle at which the amplification curve crosses the threshold. The delta Ct (ΔCt) value was then calculated for each sample, describing the difference between the Ct value

of the target gene and the Ct value of a housekeeping gene (used as a reference gene).

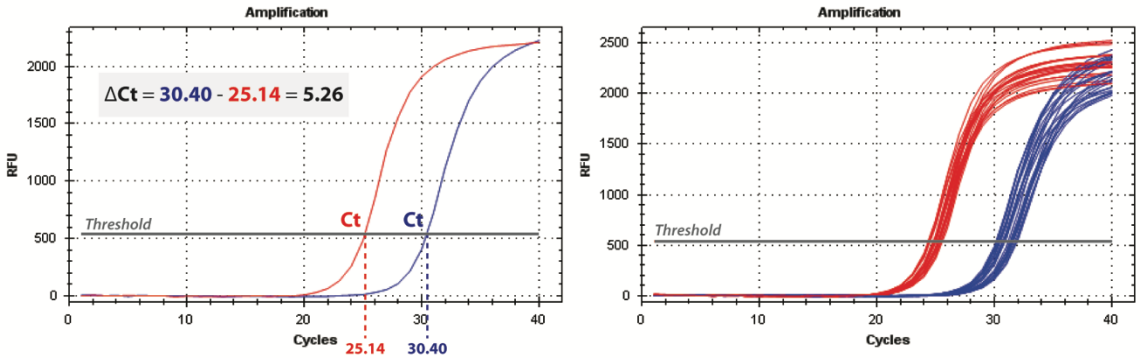


***Figure 2.7: Amplification curves. Left**: Amplification curves of a target gene (blue) and a house keeping gene (red) from the same sample. Cts were obtained from the threshold line (grey) set up within the linear segment of the curves. ΔCt was calculated as the difference between the two Ct values. **Right**:Amplification curves of an entire 96 well plate assay (blue: target gene; red: house keeping gene).*

e. Primer quality control

The primers, when designed, should have key specific characteristics: they must be complementary to the target sequence with no homo-complementarity (single primer sequence itself) or hetero-complementarity (couple of primers together). Their length should be 18-30 nucleotides, their melting temperature should be similar (determined by a guanidine-cyanidine content of about 40-60%). Primers can be designed by using freely-available online tools hosted on the *National Centre for Biotechnology Information* (NCBI) website. In this study, the primers were purchased from *Qiagen* (*RT^2 qPCR Primer* assay) and were already tested and validated by the supplier.

The melting curve indicates whether the qPCR assay produced a specific amplicon. Since SYBR Green is not specific and binds to every double-strained molecule, it is important to ensure that only one amplicon has been amplified. The qPCR assay was validated if there is only one melt peak for one couple of primers (**Fig. 2.8**).

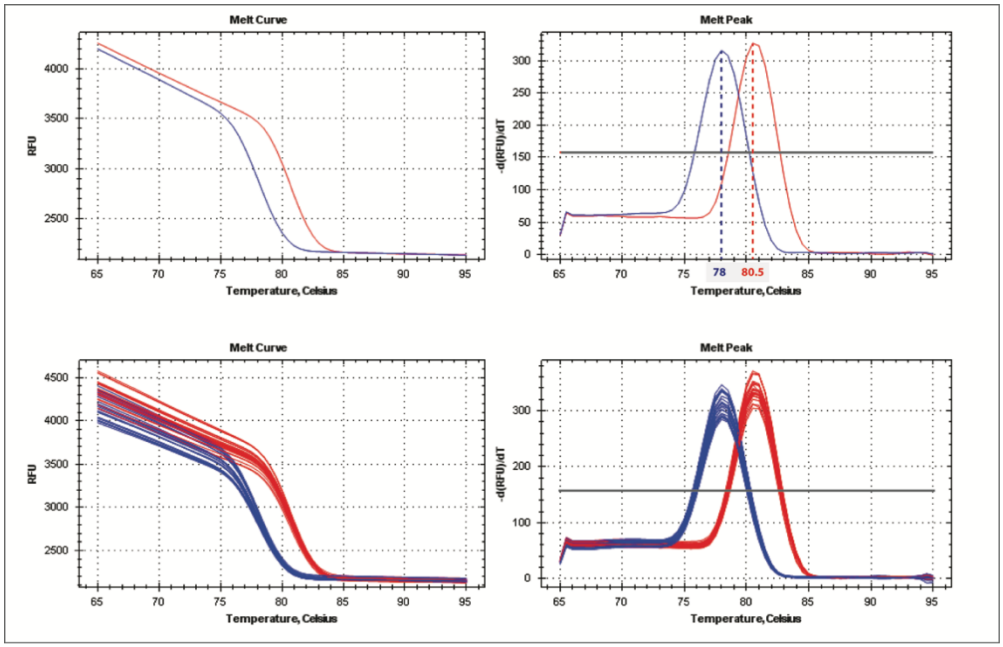


***Figure 2.8: Melting curves and melting peaks. Top**: Melting curves and peaks of a target gene (blue) and a house keeping gene (red) from the same sample. A single peak of each target is observed, validating the specific amplification of a single amplicon. The melting temperatures of the target gene and the house keeping gene are 78°C and 80.5°C respectively. Bottom: Melting curves and peaks from an entire 96 well plate assay (blue: target gene; red: house keeping gene). The specific amplification of a single amplicon per gene is validated when a single melting peak is observed for each target.*

4. In situ hybridisation

In-situ hybridisation is a method enabling the detection and quantification of single-strained nucleic sequence (i.e. mRNA) directly, in situ, on brain sections. A complementary sequence of a target mRNA (around 40 nucleotides) called an oligonucleotide probe is tailed by 3'OH incorporation of radioactive ^{35}S-dATP and then incubated with brain sections wherein it binds in a sequence-specific manner its complementary target mRNA. The probe was designed by using online tools available in free-access on the *NCBI* website.

The in-situ hybridization protocol is based on 5 consecutive steps:

Probes labelling
Probes purification
Pre-hybridisation treatment
Probes hybridisation
Post-hybridisation treatment

a. Probes labelling

Oligonucleotide probes were diluted in sterile water at a final concentration of 10ng/µl.

Probes labelling was carried-out by incorporation of ^{35}S-dATP at the 3'OH end of the oligonucleotide sequences by the Terminal deoxynucleotidyl Transferase (TdT, *Promega*). Oligonucleotides, TdT, TdT buffer, ^{35}S-dATP and sterile water were mixed at the concentrations represented in **Table 2.3** and incubated for an hour at 37°C.

Reagents/solutions	Volume (µl)
Sterile water	11.9
5X TdT buffer	4
^{35}S-dATP	1.6
Probe (10ng/ul)	2
TdT	0.5

Table 2.3: Composition of the probe labelling mix

Following the first hour of incubation, 0.5µl of TdT were added to the reaction and the mix was incubated for another hour at 37°C. The enzymatic reaction was subsequently stopped by adding 2µl EDTA (250mM), 2µl tRNA (20mg/ml) and 35.5µl Tris-EDTA buffer (1X TE). 1µl of the final mix was quantified in 2 mL of liquid scintillation cocktail (Ultima Gold*, Perkin Elmer, USA*) allowing the conversion of radioactive emissions into photons, with a scintillation counter to evaluate the total level of radioactivity.

b. Probes purification

To discard fragments of labelled and unlabelled oligonucleotide probes and free ^{35}S-dATP which would greatly decrease the signal/noise ratio, the mix was purified through a chromatography column made of Sephadex G-50 beads (*Sigma*) and TE-1X buffer. Eluent was collected in 16 tubes (500µL per tube); 2µl of each tube were then quantified in 2 mL Ultima Gold in the scintillation counter. The samples among the first eight that contain the higher radioactivity levels are those in which the labelled probe was collected. The scintillation levels allowed us to calculate the percentage of radioactivity incorporated (compared to the total radioactivity from the final probe labelling sample) which offers insight into the specific activity of the probe.

The contents of the tubes containing the probe were mixed 10% (vol/vol) 5M NaCL and 2 volumes 100% Ethanol and were precipitated overnight at -20°C. The tubes were then centrifuged at 13000g at 4°C for 1 hour, the supernatant was collected (residual radioactivity in

the supernatants was assessed) and the pellets containing the radioactive probes were dried 1 hour at RT, before being suspended in 1X TE (the radioactivity was assessed with the same protocol as described previously) and stored at -20°C.

c. Pre-hybridisation treatment

Fresh brain sections need to be processed to allow the specific hybridisation of the probes onto the targeted mRNAs. Slides containing brain sections were dried at room temperature for an hour, then, went through a succession of agitating baths starting with paraformaldehyde (PFA) 4% followed by several baths of saline sodium citrate (SSC) (**Table 2.4**).

Solutions	Incubation time
4% PFA	15 min
4X SSC	10 min
4X SSC	10 min
4X SSC + 1% Denhardt 100X	30 min
4X SSC + 1% Denhardt 100X	30 min
4X SSC	15 min
4X SSC	15 min
4X SSC + 1.33% Triethanolamine	5 min
4X SSC + 1.33% Triethanolamine +Acetic	5 min

Table 2.4: Pre-hybridisation treatment

PFA is used to fix the sections, i.e., prevent the degradation of the tissue by creating covalent bonds between molecules. The saturation of non-specific sites was mediated by the Denhardt baths which prevents any further non-specific binding of the probes onto the tissue. Triethanolamine and acetic acid contributed to the acetylation step which also minimized the formation of non-specific complexes.

The last step of Pre-hybridization was dehydration, slides were immersed 20 seconds in increased Ethanol concentration baths: 75%, 80%, 95%, 100% and were dried at room temperature for 1 hour.

d. Probes hybridisation

Probes stored at -20°C were diluted in the hybridisation buffer (**Table 2.5**) at the final

concentration of 0.5ng/40µl.

Reagents/solutions	Concentration
Sulfate Dextran	10%
Denaturated ssDNA	50ng/ml
Sarcosyl	5%
20X SSC	2X
10% SDS	0.2%
0.5M EDTA pH8	1mM
5M NaCL	300mM
Formamide deionised	50%
100X Denhardt	5X
5M DTT	2%

Table 2.5: Composition of the hybridisation buffer

40µl hybridization buffer was deposited onto each slide and covered by cover glass. Slides were placed in boxes (with wet lined cotton to maintain a constant humid environment) overnight at 42°C. The hybridisation temperature, which should be close to the melting temperature (Tm), is lowered to 42°C by Formamide deionised added to the buffer which helps lowering the melting temperature of nucleic acids by denaturing their secondary structures.

e. Post-hybridization treatment

Following the overnight hybridisation, cover glasses were carefully removed and sections were rinsed with SSC 1X. Sections were then processed through a series of post-hybridisation treatments under agitation (**Table 2.6**).

Solutions	Incubation time	Incubation temperature
1X SSC	15 min	RT
1X SSC	45 min	RT
1X SSC	15 min	45°C
1X SSC	45 min	45°C
0.1X SSC	15 min	45°C
0.1X SSC	45 min	45°C

Table 2.6: Post-hybridisation treatment

The first series of baths were crucial to remove the excess of non-binding probes then, the

45ºC baths (higher temperature than during the hybridisation) disrupted non-specific complexes.

The last step of Post-hybridization was dehydration. Slides were immersed 20 seconds in increased ethanol concentration baths: 75%, 80%, 95%, 100% and were dried at room temperature for 1 hour.

Slides were placed in an X-ray cassette and secured with tape. In a dark room, a *Kodak biomax MR* film was inserted in the cassette, the emulsion side facing the slides. The film was exposed in the dark to the slides for as long as necessary to yield signal in the linear range of the film. Exposure time was dependent upon the quality of the probe and the level of expression of the mRNA targeted but generally varied from 1 to 10 weeks. Films were revealed in a dark room. Pictures of each brain section were taken on a light table and subsequently analysed with *ImageJ software*. An area was drawn in the structure of interest wherein an optical density value was obtained. The optical density in an mRNA-free portion of the brain (i.e. a fibre tract) was defined as background and this value was subtracted to the one obtain from the area of interest to compute the relative optical density used as the dependent variable in subsequent analyses.

Data and statistical analyses

All the data are presented as mean +/- sem (standard error of mean) or individual data points and were analysed with *Statistica software* 10 (*StatSoft*). Behavioural data were subjected to repeated measures, one-way, two-ways or factorial analyses of variance (ANOVAs). Assumption for parametric analyses, namely homogeneity of variance, sphericity and normality of distribution were verified prior to each analysis with Cochran, Mauchly and Sapiro- Wilk's tests respectively. The confirmation of significant main effects and differences among individual means were analysed using the Newman-Keuls *post-hoc* test. For all analyses, the significance level was $p < 0.05$.

CHAPTER 3: BEHAVIOURAL CHARACTERISATION OF INCENTIVE HABITS AND COMPULSIVE HEROIN SEEKING

Introduction

Addictive drugs are powerful positive reinforcers. The positive reinforcing properties of addictive drugs can be measured in humans and in other animals under experimental conditions such as self-administration procedures [165, 217]. Acquisition of self-administration has been shown to be a reliable marker of these reinforcing properties [218] and drugs that are addictive in humans are volitionally self-administered by several animal species including rats [219-221], mice [222, 223], dogs [224] and non-human primates [225].

Since the first self-administration procedure in rats in the 60's (using fixed ratio schedule of reinforcement) [226], a broad range of reinforcement schedules have been developed, attempting to better operationalise the behavioural features of addiction in humans. The clinical perception of drug addiction has evolved dramatically over the past twenty years, as illustrated by the change in the clinical definition of drug addiction in the DSM. In the DSM III in the 80's, addiction was defined by the presence of tolerance and withdrawal, whereas in the current DSM 5 [139] tolerance and withdrawal are no longer necessary for addiction to be diagnosed. Rather diagnostic categorisation has progressively focused on motivational and compulsive behavioural and psychological features, including craving [139].

Interestingly these progressive changes in the clinical definition of drug addiction were introduced as a result of the emergence of stimulant addiction, especially crack cocaine, in the 80's. Individuals addicted to stimulants such as 'crack' clearly show compulsive drug seeking, but exhibit no obvious physical sign of withdrawal and a very different pattern of use as compared to opiates or alcohol addiction. While cocaine use has increased linearly since the introduction of crack, most of the research on the psychological and neurobiological substrates of addiction has focused on stimulants, leaving heroin under-investigated for several decades. Unfortunately, the current epidemic of opiate overdoses in the United States of America is a sad reminder that heroin still kills many individuals and remains the most harmful of illicit drugs [10].

However, in marked contrast with cocaine, the psychological, neural and cellular factors that contribute to the individual vulnerability to switch from controlled heroin intake to compulsive heroin seeking habits, the hallmark of the addiction developed by a small fraction of those who engage in heroin use [227, 228], are unknown. This may stem from the lack of preclinical models of compulsive heroin seeking.

The development of refined procedures in preclinical models that capture the complexity of aberrant drug-taking and drug-seeking behaviours, and the underlying neuro-psychological processes, displayed by individuals addicted to drugs has become a new frontier in the field of pre-clinical research on addiction which has hitherto only been reached for cocaine [229] (**Fig. 3.1**).

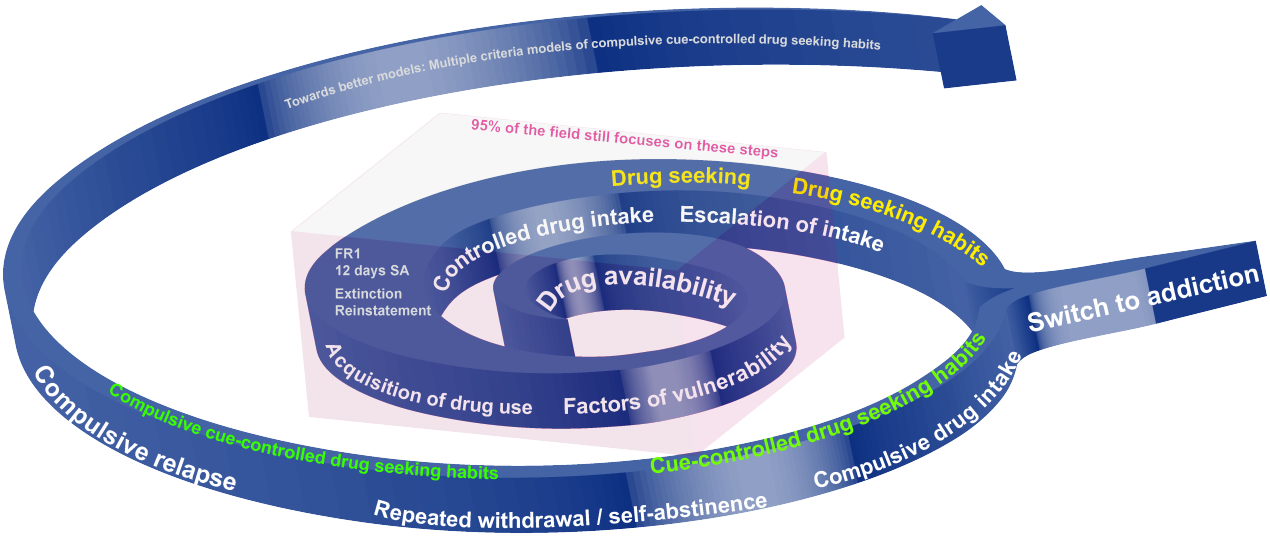


Figure 3.1: The vicious spiral of drug addiction depicting the various behavioural and psychological stages and how models map on it. *95% of the addiction research field is focusing on the early stages of the development of drug addiction such as drug availability, acquisition of drug use, adaptations following short-term access to the drug (pink cube). To better operationalise the diagnostic critera of drug addiction, our community has to implement more sophisticated procedures that catpure the compulsive nature of drug intake/seeking (or relapse) or the development of compulsive drug seeking habits .*

Addiction is considered the end-point of a series of within- and between-systems adaptations in the brain, including sensitisation to the incentive properties of the drug [140] and the development of hedonic allostasis associated with a shift from positive to negative reinforcement [66, 111, 141]. However, these adaptations eventually influence the neural and associated psychological mechanisms governing instrumental behaviour, such that drug use that is initially goal-directed becomes habitual, triggered and invigorated by both exteroceptive and interoceptive cues, and eventually compulsive, i.e. maintained despite negative consequences

[230]. Addiction therefore relies on the aberrant recruitment of Pavlovian and instrumental mechanisms which interact to generate maladaptive behaviour [65, 231, 232].

In psychological terms, instrumental conditioning results from the contingency between an action and the delivery of a reinforcer (lever press = drug). Pavlovian conditioning results from the contingency between a stimulus, initially neutral and a reinforcer, through repeated non-contingent exposures the stimulus becomes associated with the unconditioned stimulus (reinforcer). It is then a Conditioned stimulus (Cs) that predicts the immediate availability of the reinforcer, but importantly has also acquired sensory-specific properties and motivational value in its own right [233, 234].

Instrumental performance can be controlled by two processes that are psychologically and neurobiologically dissociable, namely goal-directed (mediated by action-outcome A-O associations) or habitual performance (mediated by stimulus-response S-R associations) (**Fig. 3.2**).

The distinction between A-O and S-R control over instrumental responding relies on the sensitivity of the instrumental response measured under extinction after either a devaluation of the outcome or a degradation of the contingency. When instrumental performance is goal-directed, it is preceded by a representation of the motivational value of the reinforcer, i.e. the drug and the contingency between the action and the outcome (**Fig. 3.2B**); therefore, if the value of the outcome is diminished, instrumental performance to attain it will drop. If the response is under S-R control, it will be impervious to the same manipulation of the outcome or the contingency [235, 236].

Thus, the acquisition of drug seeking, both for alcohol for which the instrumental response is dissociated from the consummatory response [237, 238], and for cocaine, as measured in a seeking-taking chained schedule of reinforcement [239], is controlled by A-O mechanisms. However, after extended training instrumental drug seeking becomes established as a habit in that is becomes impervious to manipulations of the motivational value of its immediate consequence (outcome in the case of alcohol, access to an extinguished taking lever in the case of the seeking-taking task for cocaine) [238, 239] (**Fig. 3.2C**) [for review, 145, 240].

At the neural systems level, goal-directed and habitual control over behaviour have been

shown to rely on dissociable corticostriatal networks [241]. Chiefly, goal-directed control over behaviour devolves to the orbitofrontal cortex, the prelimbic cortex, the ventral striatum (nucleus accumbens, Acb) and the posterior dorsomedial striatum (pDMS) whereas habits have been shown to depend on the infralimbic cortex, and the anterior dorsolateral striatum (aDLS). This will be further discussed in more detail in chapter 5.

However, in real life; instrumental responses are not divorced from pavlovian cues, and it has been shown that pavlovian influence over instrumental performance is of greater magnitude when the latter is governed by S-R, as compared to A-O, associations [242]. Therefore, although S-R behaviour does not rely on a representation of the motivational value of the goal, habits remain sensitive to the motivational influence of Pavlovian CSs.

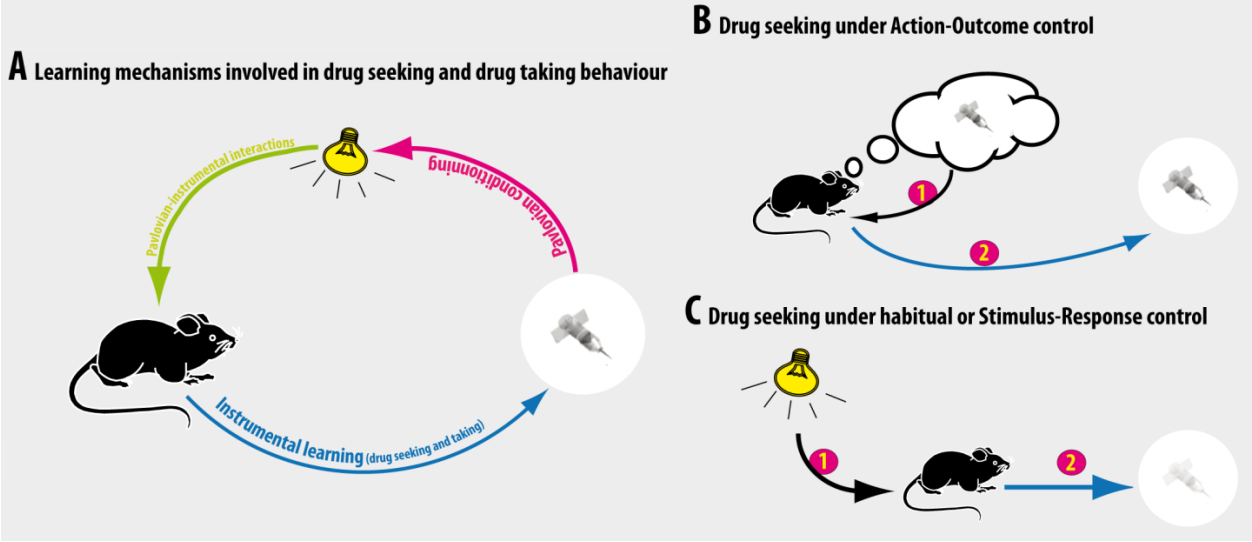


Figure 3.2: Psychological processes involved in the control over instrumental responding. ***A)*** *Schematic representation of associative pavlovian learning processes (pink arrow) occuring over the course of the acquisition of drug seeking behaviour (instrumental conditioning, blue arrow). The green arrow represents the interactions between pavlovian and instrumental learning mechanisms.* ***B)*** *When drug taking behaviour is under the control of A-O associations, instrumental performance is initiated following the explicit representation of the motivational properties of the drug and the relationship between the action and the outcome. Such A-O associations control seeking and taking behaviour during the early stages of training in drug self-administration and subsequent drug seeking behaviour.* ***C)*** *When drug taking is habitual (under the control of S-R associations), the instrumental response is triggered with no preceding representation of the motivational value of the outcome, it is dissociated from any representation of the motivational properties of the drug itself. Habitual seeking behaviour emerges after a protracted training under conditions in which the contingency between action and outcome delivery is low (seeking-taking chained, fixed interval or second order schedules of reinforcement) [after 243].*

Thus, drug seeking behaviour stems from interactions between Pavlovian and instrumental mechanisms, both of which hijacked by addictive drugs so that well established drug seeking behaviour is instantiated as a maladaptive habit which is reinforced and invigorated by Pavlovian associated conditioned stimuli (CSs), acting as conditioned reinforcers, bridging delays to

reinforcement [146, 244]. As presented previously, such intricate interactions between Pavlovian and instrumental mechanisms involved in drug seeking are best operationalised in second order schedules of reinforcement [216]

It has been suggested [7, 144, 145] that when drug seeking habits are invigorated by conditioned reinforcement over extended periods of time, specific psychological mechanisms and underlying adaptations within the corticolimbic circuitry [146, 245], are engaged to trigger the development of incentive habits whereby the motivational value of the US is transferred to the response through the conditioned reinforcer mediating a second order interdimensional conditioning (**Fig. 3.3**). These incentive habits, aberrantly bridging positive or negative emotional properties of Pavlovian cues with the S-R associations subserving drug seeking habits, have been suggested to be a gateway towards the development of compulsivity [145, 243].

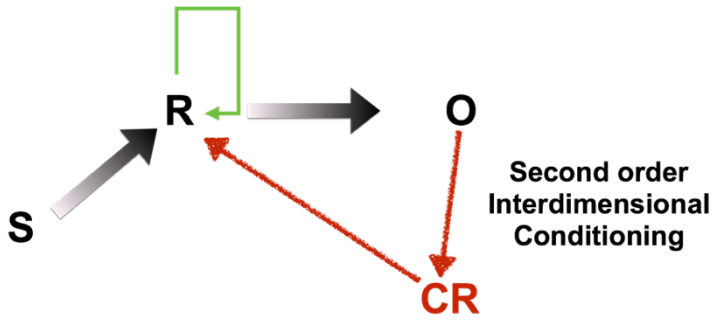


Figure 3.3: Psychological model of incentive habits. *Upon protracted exposure to conditioned reinforcement, the motivational value of the outcome that is represented in the CS, is, when utilised as a CR, transferred to the response even if that response is mediated by S-R associations. Thus, the CR offers a bridge, whereby the motivational value of the outcome, otherwise dissociated from a representation of the response, is transferred to it through a so-called second-order interdimensional conditioning process.*

According to this incentive habit theory, recently developed by Belin and colleagues [144, 145, 246], the aberrant motivation seemingly directed towards the drug displayed by drug addicts when they are engaged in drug seeking may represent an aberrant engagement of instrumental responding, the goal of which is to express the drug seeking habit, rather than the drug itself. Thus, in psychological terms, if something in the environment impinges on the behavioural manifestation of their drug seeking habit, they may well recruit a new overarching cognitive schemata under the control of an action-outcome association, the representation of which is not about the motivational properties of the drug, but the opportunity to perform the drug seeking habit response. In that context, strategies that aim at reducing the motivational or reinforcing properties of the drug would not be effective at reducing the seeking response. **This opens avenues for specific predictions to be tested here, such as whether rats displaying incentive habits for heroin would indeed aberrantly engage in responding in the anticipation of, or after**

exposure to, punishment, which would hamper the 'natural' expression of their habit.

The neurobiological underpinnings of incentive habits have started to be delineated using causal functional manipulations of the corticostriatal circuitry in rats trained to seek cocaine or heroin under second order schedules of reinforcement. Thus, well-established performance under a second order schedule of reinforcement for both cocaine and heroin results in the devolvement of control over dopaminergic mechanisms in the aDLS (confirming that drug seeking behaviour becomes habitual) [146]. Interestingly, in humans, dopaminergic activation in the DLS has been shown to be triggered by cocaine-related pictures in recreational users [133], thereby suggesting that this structure which has also been shown to support compulsive cocaine seeking in rats [247, 248] and is associated with craving in individuals addicted to cocaine [132] is recruited before the onset of addiction and is therefore not sufficient to account for the development of compulsivity.

Compulsivity can be characterised as the persistence of performing an action without specific purpose and despite adverse consequences and it can be operationalized in animal models as the persistence of instrumental responding in the face of electric foot-shock-induced punishment [192, 193].

Since the first study conducted on avoidance of nociceptive stimuli in the context of behaviours motivated by various drives [249], electric foot-shocks have been used on rodents in pre-clinical models of addiction [192, 193, 199, 250-254].

When foot-shocks are presented contingently with an instrumental response, under conditions which do not enable the establishment of counter-conditioning [255], they are used to operationalise the persistence of responding despite negative consequences. Using a preclinical model of addiction factoring in several behavioural features reminiscent of the diagnostic criteria of addiction [139], Belin and colleagues [192, 193] showed that, after more than 70 days of training, a subpopulation of rats trained to self-administer cocaine under a fixed ratio 5 schedule of reinforcement, was prone to continue self-administering cocaine despite being punished by mild foot-shocks, and therefore fulfilled a hallmark feature of addiction, namely compulsivity. However, that procedure suffers from a major limitation, in that drug addicts display the compulsive nature of their behaviour when they are engaged in foraging for

the drug, not when they are actively taking the drug.

Seeking (preparatory) and taking (consummatory) responses are psychologically dissociable. Therefore, it is important, spatially and temporally to dissociate them in behavioural tasks operationalising compulsivity. Pelloux and colleagues [252] used a probabilistic punishment embedded in a seeking-taking task, to identify rats that are more prone than others to maintain their seeking responses despite the risk of receiving a punishment, demonstrating compulsive drug seeking, and not taking.

While compulsive cocaine and alcohol seeking behaviour has been successfully operationalised in rats by the punished seeking-taking task [197, 199, 200, 251], there is currently no available model of compulsive heroin seeking behaviour. This may arise from the fact that the punished seeking-taking tasks do not include long periods of drug-free seeking responses, and therefore measure resistance to punishment whilst the drug is on board, which is incompatible with the well documented analgesic properties of opiates.

Indeed, in the punished seeking-taking task, developed in Pr. Everitt's lab, rats are trained to self-administer cocaine under a chain schedule whereby responding on a seeking lever gives access, following a variable interval, to another lever (taking lever), a press on which results in a drug infusion. Once animals display stable levels of responding, a random punishment schedule is introduced wherein 50% of seeking responses are punished (mild foot-shock) while 50% of responses give access to the taking lever. This protocol is very relevant for probing the compulsive component of cocaine or alcohol seeking behaviour but does not meet the expected criterion to assess the contribution of incentive habit to compulsivity, especially in the context of heroin self-administration. Indeed, the chain schedule implemented in this model, facilitates stable levels of seeking behaviour following protracted training. However, the seeking responses are not reinforced by contingent presentation of drug-associated stimuli and therefore may not support the emergence of incentive habits. Furthermore, the drug seeking period follows a random interval schedule that often results in a drug infusion soon after the beginning of the session. This is arguably a suboptimal measurement of drug seeking behaviour, as it should be measured in a drug-free state over prolonged periods of time.

Finally, and most importantly, in this task, individuals are exposed to the drug prior to

receiving an aversive stimulus, which, given the analgesic properties of heroin represents a major bias.

Thus, nothing is known about the psychological and neural substrates of inter-individual vulnerability to develop compulsive heroin seeking habits. Importantly, it remains unknown whether the establishment of incentive habits for heroin contribute to maladaptive drug seeking behaviour.

Therefore, a novel model of compulsive heroin seeking behaviour was designed and developed over the course of this research project that capitalises on the second order schedule of reinforcement, which has been shown to facilitate the emergence of aDLS-dopamine dependent cue-controlled drug seeking, or incentive, habits [146]. For this, punishments were introduced within each FI-15 interval of daily sessions so as to test inter-individual vulnerability to develop compulsive heroin seeking habits.

To further investigate the specific neurobiological correlates of incentive habits as compared to habitual drug seeking (as measured in FI15 schedules of reinforcement), a group of rats was trained under the same punishment schedule following a similar history of heroin seeking, but under a FI-15 schedule of reinforcement.

The development of a novel model of heroin addiction with heuristic value with regards to the human situation would also enable the investigation of the neurobiological substrates of compulsive drug seeking behaviours and identify the underpinnings of incentive habits.

Following the development and validation of this new model of individual vulnerability to develop compulsive drug seeking behaviour, I tested the hypothesis that the behavioural outcomes observed with this heroin model were generalizable to cocaine. Thus, I carried out a similar experiment with cocaine as a reinforcer, and parametrically compared compulsive drug seeking behaviour between rats exposed to either heroin or cocaine.

Materials and methods

1. General experimental design

Sprague Dawley rats (n = 120) were subjected to intravenous catheterisation as described in

chapter 2. Following five post-operative days of recovery, rats were trained to self-administer either heroin (0.04mg/infusion) (n = 72) or cocaine (0.25mg/infusion) (n = 48) in the operant chambers previously described.

Rats acquired self-administration under a Fixed Ratio 1 (FR-1) schedule of reinforcement until they reached a significantly higher level of responding on the active lever than on the inactive lever thereby showing discrimination and stable rate of injections. Following acquisition of instrumental learning, rats were then exposed to Fixed Interval schedules with increasing durations so that they acquired drug seeking over drug-free periods of increasing duration (from 1 minute (FI-1) to 15 minutes (FI-15), over six consecutive sessions). Rats were trained under a FI-15 schedule of reinforcement for three days in order to measure their stable level of drug seeking behaviour prior to the introduction of the contingent CS presentations. This will enable the quantification of the magnitude of conditioned reinforcement when rats will subsequently be trained under a second order schedule of reinforcement (SOR).

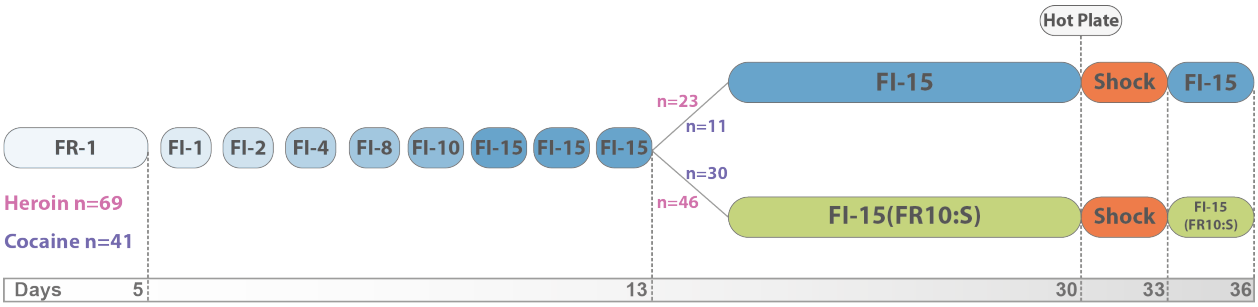


Figure 3.4: Schematic representation of the timeline of the heroin self-administrations experiment. *Rats were trained to self-administer heroin (n = 69) or cocaine n = 41) under FR-1 for 5 days, then FI-1 to FI-15 schedule of reinforcement. Following these sessions, in the compulsive drug seeking procedure, rats (heroin: n = 46; cocaine: n = 30) were trained 17 days under a second order schedule of reinforcment (SOR groups) while other subjects (heroin: n = 23; cocaine: n = 11) were maintained under FI-15. Pain thresholds were measured using a hot-plate test, and compulsivity was measured over 3 sessions during which electric foot-shocks were introduced. Rats recovered their drug seeking behaviour after cessation of punishment under the same schedule of reinforcement as before (FI-15 or 2nd order schedule).*

Subsequently, rats were trained under a second order schedule of reinforcement (SOR) (n = 48 for heroin and n = 36 for cocaine) for 17 daily sessions, under conditions previously shown to facilitate the development of incentive habits [146, 202]. The remaining rats were maintained under a FI-15 schedule of reinforcement (n = 24 for heroin and n = 12 for cocaine). This experimental design enabled the investigation of the potential contribution of conditioned reinforcement (hence incentive habits) to the qualitative and quantitative nature of the development of compulsive seeking behaviour. Following these training sessions, compulsive

drug seeking behaviour was assessed by measuring the persistence of seeking responses despite contingent mild electric foot-shocks (**Fig. 3.4**).

Thus, for three daily sessions under either FI-15 or SOR, rats were punished only when they were actively engaged in foraging for the drug, namely during the second half of each 15 minute interval. During these last 7 min of each interval, mild electric foot-shocks (1 second, 0.45mA) dispensed by a scrambler (*Med Associate St. Albans, USA*) connected to the grid floor of the operant boxes were delivered every 16th lever press (**Fig. 3.5**). Punishing responding only during the second part of each interval ensured animals did not receive electric foot-shocks immediately following a drug infusion, which could result in counter-conditioning or at least decrease the aversiveness of the shock due to the analgesic properties of heroin.

Each aversive stimulation was paired with a cue light stimulus located on the top middle part of the wall boxes (independent from those paired with drug infusions). Shocks were delivered every 16th lever press in order to minimise the likelihood that the drug paired CSs and shocks would co-occur and potentially lead to a total extinction of their drug seeking behaviour.

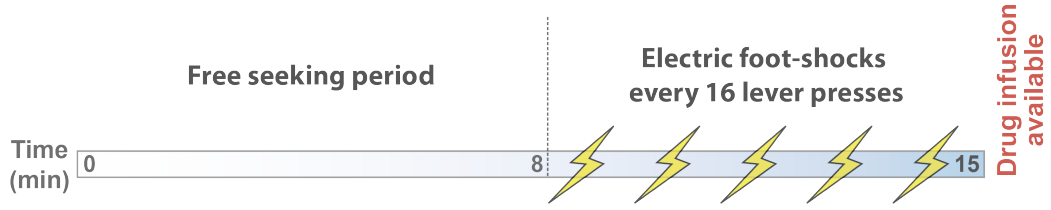


Figure 3.5: Schematic representation of a punished 15 min interval. *For each schedule, namely FI-15 or SOR, from the beginning of the interval to the 8th minute, animals were free to seek drug. However, during the last 7 min (from the 8th to the 15th minute) electric foot-shocks were introduced contingently to each 16th lever press. Upon completion of the interval, the 1st or 10th active lever press (for SOR group and FI15 group, respectively) resulted in a drug infusion.*

To ensure that potential differences in resistance to punishment were not attributable to a differential pain threshold, rats were subjected to a hot plate test prior to shock sessions. Six hours following the 17th drug self-administration session under either FI-15 or SOR, rats were placed on a hot plate (*Ugo Basile, Gemonio, Italy*) set up to remain at a stable temperature of 52ºC. The time elapsed before apparition of pain-associated behaviours, including paw-licking and jumping, was measured and considered a direct indicator of pain threshold [256]. Rats were then immediately removed from the hot plate and returned to their home cage.

Following three daily punished sessions, rats were re-exposed to three baseline sessions (FI-

15 or SOR) to investigate their ability to recover their initial drug seeking behaviour, or to display long-term behavioural adaptations to successive punishment sessions. The very last session was a drug-free session during which rats were seeking heroin for 15 minutes. The session was stopped after 15 minutes and rats were returned to their home cage prior to being sacrificed 45 minutes later. Brains were harvested for in situ hybridisation and qPCR assays as previously described.

Three rats from the heroin group (FI-15 group: n = 1, SOR group: n = 2) and seven (FI-15 group: n = 1, SOR group: n = 6) from the cocaine group were excluded due to loss of catheter patency.

2. Data and statistical analysis

The maintenance of drug seeking despite foot-shocks was calculated as the percentage of active lever presses over the 3 shock sessions relative to the 3 pre-shock sessions.

Post-shock recovery was assessed as the level of responding displayed over the last two post-punishment sessions as compared to the last two punished sessions.

A K-means cluster analysis on the number of shocks received in the first interval of the last two shock sessions was carried out to identify non-overlapping populations of rats stratified on their ability to cope with punishment while engaged in drug seeking [200].

Considering the relatively low levels of responding of the rats in the cocaine SOR group during the first interval of the baseline prior to the shock sessions (as described and discussed, respectively in the next sections), the occurrence of shocks was very low. The segregation of the population relative to compulsivity levels by cluster analysis was therefore not relevant considering the size of the population and the limited number of shocks each individual received. Thus, for consistency, the parameter variable chosen to segregate the population was the same as the one used for heroin on which cluster analysis was carried-out, i.e. the sum of shocks received in the first interval of the last two punished sessions. This variable follows a tri-modal distribution (see **Fig. 3.7**), and was subjected to a median split of the population (median = 2) to obtain high compulsive subjects (HC, n = 6) receiving at least 3 or more shocks and low compulsive animals (LC, n = 24) receiving 2 or less shocks. As the variable was non-continuous, this method enabled the segregation of the population in two non-overlapping populations such

as type-two errors were avoided. The size of the population and the distribution narrowed around a limited number of shocks prevented the characterisation of a third population (namely intermediate compulsive subjects).

Behavioural data were subjected to repeated measures analysis of variance (ANOVA), where sessions, levers (active/inactive) were used as within-subject factors and groups used as between-subject factors. Resistance to punishment and recovery were analysed by factorial ANOVAs.

Results

After protracted training under either a FI-15 or SOR schedule of reinforcement, rats, eventually displayed a greater rate of responding temporally close to the delivery of the reinforcer, i.e. during the last 7 minutes of the 15-minute interval *[Heroin: main effect of block: $F(1.67) = 60.302$, $p < 0.0001$ / Cocaine: main effect of block: $F(1.39) = 29.283$, $p < 0.0001$]* (**Fig. 3.6**). This specific dichotomy in the temporal distribution of responding enabled the identification of the last 7 min of the interval as the period during which seeking behaviour would be punished (see Methods).

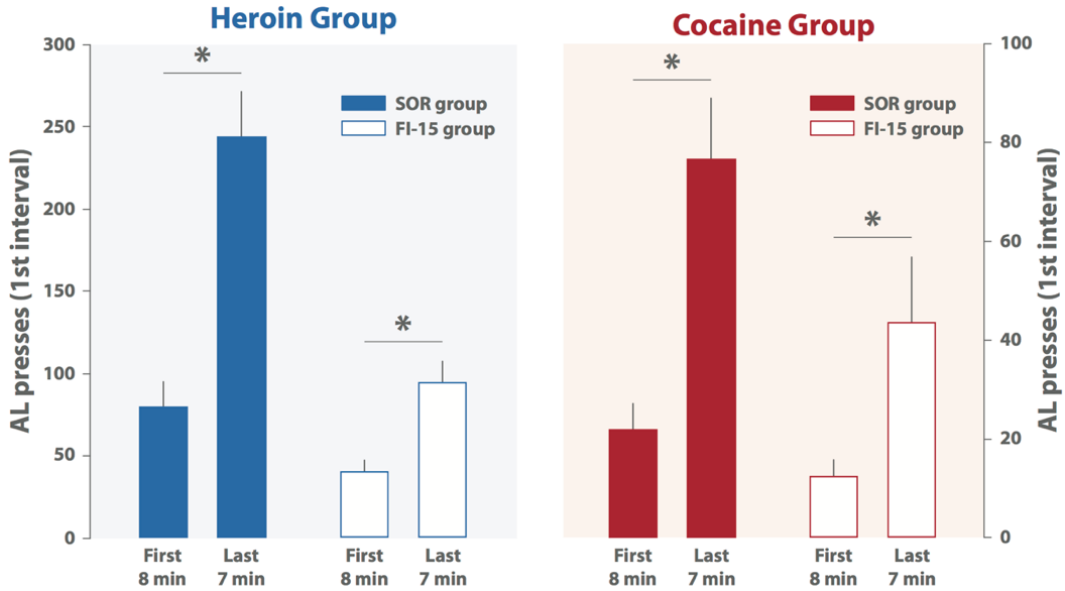


Figure 3.6: Temporal distribution of active lever during the first 15 min interval over the 3 pre-shock baseline daily sessions.*Irrespective of the nature of both the reinforcer and the schedule of reinforcment, rats displayed significantly higher level of responding on the active lever during the last 7 min of the first interval during the three pre-shock baseline sessions, in agreement with what had been shown by Arroyo and colleagues [201]. This pattern of responding was used to define the temporal window within which to punish ongoing seeking responses (i.e. the last 7 min). [*: $p < 0.05$]*

Optimised conditions were therefore identified to probe the influence of "incentive habits" for heroin over the maintenance of drug seeking despite adverse consequences.

1. Heroin experiment

a. Comparative behavioural characterisation of rats responding for heroin under FI15 and SOR prior to, during and following punishment: whole session

Rats (n = 69) acquired heroin self-administration under a FR-1 schedule of reinforcement for 5 days, as revealed by an increase in active lever presses as compared to inactive lever presses over time *[main effect of lever: $F(1,67) = 58.49$, $p < 0.0001$; session: $F(4,268) = 4.39$, $p < 0.01$; and lever x session interaction: $F(4,268) = 15.416$, $p < 0.0001$]* (**Fig. 3.7A**). No difference was observed between the rats that will be allocated to the SOR or the FI-15 group *[main effect of group: $F(1,67) < 1$ and group x lever interaction: $F(1,67) < 1$]*.

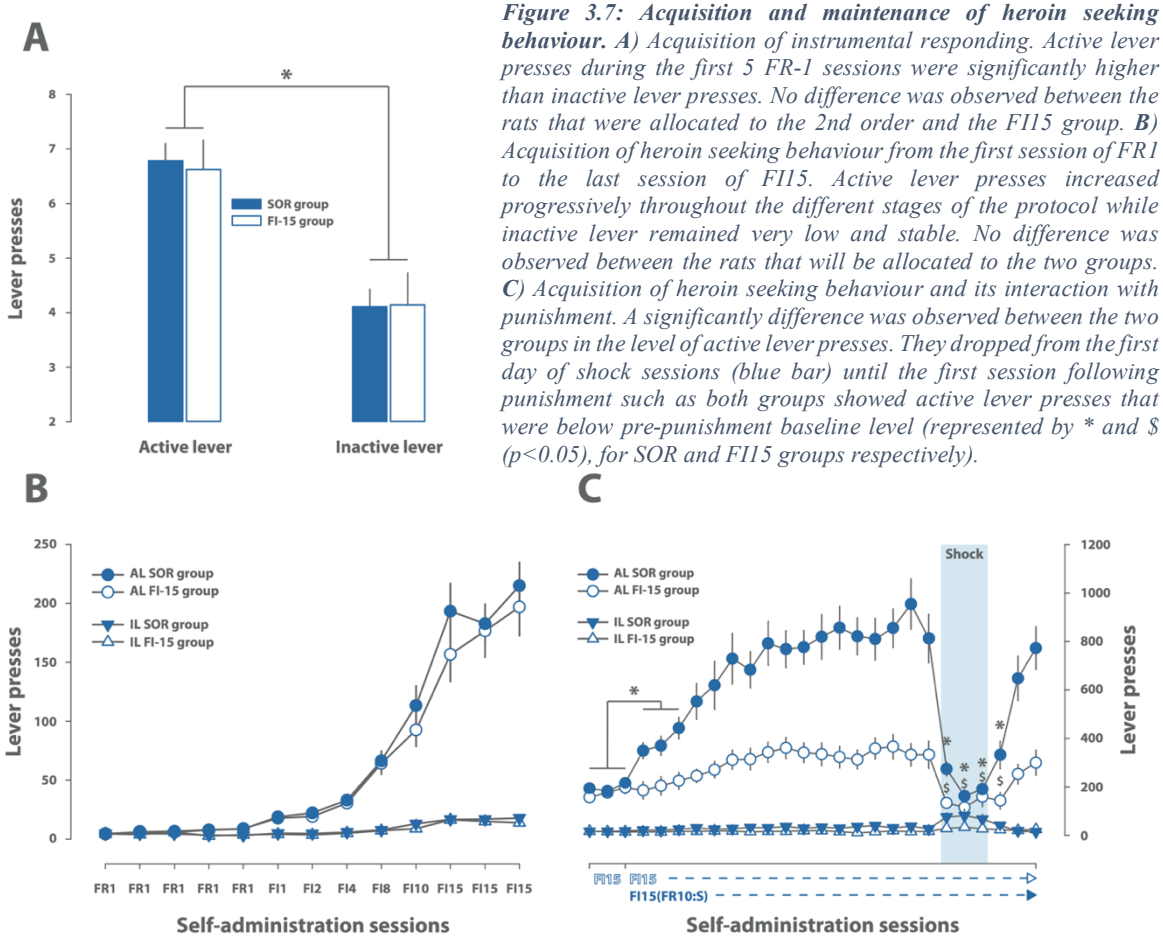


Figure 3.7: Acquisition and maintenance of heroin seeking behaviour. *A) Acquisition of instrumental responding. Active lever presses during the first 5 FR-1 sessions were significantly higher than inactive lever presses. No difference was observed between the rats that were allocated to the 2nd order and the FI15 group.* ***B****) Acquisition of heroin seeking behaviour from the first session of FR1 to the last session of FI15. Active lever presses increased progressively throughout the different stages of the protocol while inactive lever remained very low and stable. No difference was observed between the rats that will be allocated to the two groups.* ***C****) Acquisition of heroin seeking behaviour and its interaction with punishment. A significantly difference was observed between the two groups in the level of active lever presses. They dropped from the first day of shock sessions (blue bar) until the first session following punishment such as both groups showed active lever presses that were below pre-punishment baseline level (represented by * and $ ($p<0.05$), for SOR and FI15 groups respectively).*

Rats were then trained to seek heroin for increasing periods of time under fixed interval schedules of reinforcement which resulted in a robust increase in active lever presses from FI-1 to FI-15 (**Fig. 3.7B**) *[main effect of session: $F(12,804) = 106.40$, $p < 0.0001$; lever: $F(1, 67) = 59.03$, $p < 0.0001$ and session x lever interaction: $F(12,804) = 99.40$, $p < 0.0001$]*. No differences were observed between FI-15 and SOR groups in their levels of responding over the acquisition of drug seeking under fixed intervals of increasing duration (i.e. from FR-1 to FI-15 schedules of reinforcement) *[main effect of group: $F(1, 67) < 1$ and group x session interaction: $F(12,804) < 1$]* (**Fig. 3.7B**).

Introduction of the CSs, presented contingently upon every tenth lever press resulted in the SOR group in an immediate and pronounced increase in active lever presses compared to the FI-15 group, as shown in the first 3 post FI-15 sessions *[main effect of group: $F(1,67) = 5.4729$, $p < 0.05$; block: $F(1,67) = 42875$, $p < 0.0001$; and group x block interaction $F(1,67) = 23.525$, $p < 0.0001$]* (**Fig. 3.7C**). This effect was persistent but increased over sessions [main effect of group: $F(1,67) = 15.26$, $p < 0.001$; session: $F(19,855) = 29.50$, $p < 0.0001$; lever: $F(1,45) = 105.01$, $p < 0.0001$; session x lever interaction $F(19,855) = 27.31$, $p < 0.0001$; and session x group interaction: $F(19,1273) = 7.31$, $p < 0.0001$], suggesting that heroin seeking behaviour became progressively invigorated by the CSs acting as reinforcers (**Fig. 3.7C**). Over the same period of 17 sessions, the FI-15 rats, that were maintained under FI-15 schedule of reinforcement, also displayed a progressive increase in active lever presses over time [main effect of session: $F(19,418) = 9.54$, $p < 0.0001$; lever: $F(1,22) = 70.53$, $p < 0.0001$ and session x lever interaction: $F(19,418) = 9.87$, $p < 0.0001$], albeit to a lesser extent than SOR rats (**Fig. 3.7C**).

Punishment of the ongoing drug seeking responses resulted in a marked decrease in active lever presses both in the FI15 and SOR groups *[main effect of session: $F(25,1675) = 7.03$, $p < 0.0001$; group: $F(1.67) = 15.695$, $p < 0.001$ and session x group interaction: $F(25,1675) = 7.0312$, $p < 0.001$]*. Post- hoc analysis confirmed that active lever presses were significantly lower during shock sessions than during the pre-shock sessions, and that they remained lower even during the first post-shock session (all ps < 0.05) (**Fig. 3.7C**).

b. Characterisation of heroin seeking during the first drug-free interval

Focusing on the SOR group, the introduction of contingent CSs presentation had a strong impact on heroin seeking during the first drug-free 15 min interval *[main effect of session: $F(16,720) = 32.40$, $p < 0.0001$]* (**Fig. 3.8A**).

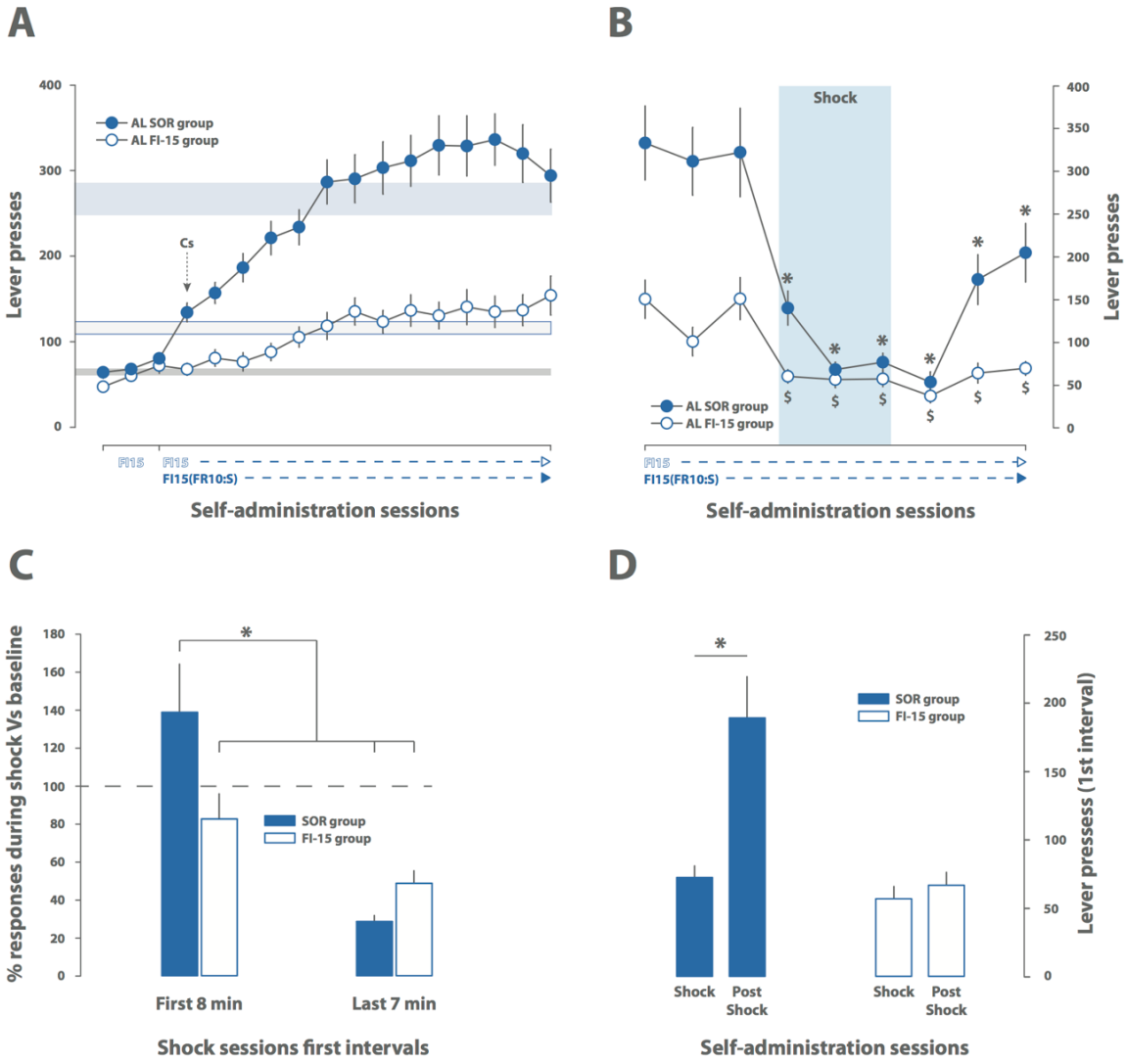


Figure 3.8: Heroin seeking behaviour displayed in the first 15 min drug-free interval. ***A****) Active lever presses during the first interval increased progressively over time both in the FI15 and the SOR groups. The average of active lever presses displayed by the SOR group once the CS was introduced (blue horizontal bar) was significantly higher than during the three preceding FI15 sessions (grey horizontal bar) and also significantly higher than those displayed by the FI15 group (grey blue-framed horizontal bar).* ***B****) Active lever presses during the first interval over the 3 pre-shock, the 3 shock (blue bar) and the 3 post-shock sessions. The average of active lever presses displayed by both groups during shock sessions were significantly lower than during baseline.* ***C****) Percentage of active lever presses over the 3 shock sessions compared to the 3 pre-shock sessions during the first unpunished 8 min and the last punished 7 min period of the first heroin seeking interval. SOR rats displayed a higher rate of responding during the first 8 min of the shock sessions than baseline sessions. Shock presentation during the last 7 min led to a decrease in active lever presses compared to the pre-shock level of responding for both group.* ***D****) Average AL presses during the last 2 shock sessions and the last 2 post-shock sessions. SOR rats increased their level of responding once punishment was terminated but FI-15 rats maintain their inhibited behaviour. [* and $: $p<0.05$]*

The average of active lever presses displayed by the SOR rats once the CS was introduced (blue horizontal bar *(266.64 ± 17.50)*) was significantly higher than the one these rats displayed during the three FI-15 sessions (grey horizontal bar *(65.18 ± 4.23)*) and the one displayed by the FI-15 group (blue-framed grey horizontal bar *(116.25 ± 7.13)*) *[main effect of group: $F(1,67) = 22.72$, $p < 0.0001$; and group x session interaction: $F(16,1072) = 7.00$, $p < 0.0001$]* (**Fig. 3.8A**).

Active lever presses performed during the first 15 min interval of each of the 3 pre-shock sessions, the 3 shock sessions (blue bar) and the 3 post-shock sessions are shown in **Fig. 3.8B**. As observed for the entire session (**Fig. 3.7C**), active lever presses dropped dramatically upon introduction of shocks in both groups and remained significantly lower than those displayed during baseline over post-shock sessions, a long lasting effect that was not observed for the whole sessions (**Fig. 3.8B**) *[main effect of session: $F(8,536) = 19.10$, $p < 0.0001$: group: $F(1,67) = 10.64$, $p < 0.01$; and session x group interaction: $F(8,536) = 4.86$, $p < 0.0001$]*.

Nevertheless, following the termination of punishment, SOR rats displayed a marked recovery in their drug seeking behaviour. Active lever presses increased over the three post-shock sessions in SOR rats, but not in FI15 rats which displayed similar levels of responding as those observed during punishment sessions. Such differential response to punishment was further supported by the comparison of the number of active lever presses displayed by SOR and FI15 rats during the last 2 post-shock sessions compared to the last 2 shock sessions *[main effect of group: $F(1,67) = 6.8467$, $p < 0.05$; block: $F(1,67) = 11.707$, $p < 0.01$ and group x block interaction: $F(1,67) = 8.3593$, $p < 0.01$]* (**Fig. 3.8D**). Thus, SOR rats were more prone to resume heroin seeking after an episode of protracted punishment as compared to FI15 rats. As I will discuss later, this may reflect the contribution of incentive habits to compulsive relapse, in that these rats are not capable of integrating the negative consequences of past punishment. This suggests that incentive habits do not facilitate resistance to punishment per se, but they may contribute to an increased propensity to relapse despite having been exposed to adverse consequences.

This was further supported by the analysis of the temporal distribution of drug seeking during the punishment sessions in SOR and FI15 rats. Indeed, while the maintenance of heroin seeking despite electric foot-shocks was calculated as the percentage of active lever presses over the 3 shock sessions relative to the 3 pre-shock sessions, punishment did not occur for the entire

duration of the interval.

FI15 rats behaved as expected in the face of punishment. Thus, active lever presses were markedly decreased by punishment not only while shocks were presented, i.e., during the last 7 min of the session, but also before, i.e. during the first 8 non-punished minutes. This revealed that punishment resulted in a general decrease in responding over the entire first 15 min interval. In marked contrast, SOR rats, while decreasing drug seeking responses during the punished period of the first interval, displayed an increase in responding during the first 8 minutes as compared to baseline. This increase of responding in anticipation of the occurrence of punishment was significantly higher than responding observed during the first 8 minutes for the FI15 group and also higher than the responding rate during the last 7 minutes for both groups *[main effect of block: $F(1,67) = 16.223$, $p < 0.001$; group effect: $F(1,67) < 1$ and block x group interaction: $F(1,67) = 4.563$, $p < 0.05$]* (**Fig. 3.8C**), thereby suggesting that incentive habits trigger an aberrant engagement in drug seeking in anticipation of the loss of the opportunity freely to respond.

2. Cocaine experiment

a. Comparative behavioural characterisation of rats responding for cocaine under FI15 and SOR prior to, during and following punishment: whole session

Rats (n = 41) acquired cocaine self-administration under a FR-1 schedule of reinforcement and met the criteria based on the discrimination between the two levers within five days, as evidenced by a selective increase in active lever presses over time *[main effect of lever: $F(1,39) = 44.194$, $p < 0.0001$; session: $F(4,156) = 2.1937$, $p>0.05$; and lever x session interaction: $F(4,156) = 16.243$, $p < 0.0001$]* (**Fig. 3.9A**). No difference in the acquisition of cocaine self-administration was observed between the rats allocated to the SOR and the FI-15 group *[main effect of group: $F(1,39) < 1$; and group x lever interaction: $F(1,39) = 1.2454$, $p>0.1$]*.

Rats were then subjected to FI schedules with increasing duration culminating in 3 sessions of FI-15 prior to the introduction of the SOR in this group. As observed in the heroin experiment, active lever presses increased from FI-1 to FI-15 (**Fig. 3.9B**) *[main effect of session: $F(12,468) =$*

33.834, p < 0.0001; lever: F(1,39) = 55.476, p < 0.0001 ; and session x lever interaction: F(12,468) = 35.648, p < 0.0001]. No differences were observed between the two experimental groups until the introduction of contingent CS presentations in the SOR group *[main effect of group: F(1,39) = 1.0434, p>0.1]* (**Fig. 3.9B**).

Subsequently, the FI-15 rats showed a similar pattern of responding to the animals in the heroin experiment. The number of active lever presses progressively increased over time until the shock sessions *[main effect of session: F(19,190) = 8.3499, p < 0.0001 ; lever: F(1,10) = 31.129, p < 0.001 and session x lever interaction: F(19,190) = 5.9089, p < 0.0001]* (**Fig. 3.9C**).

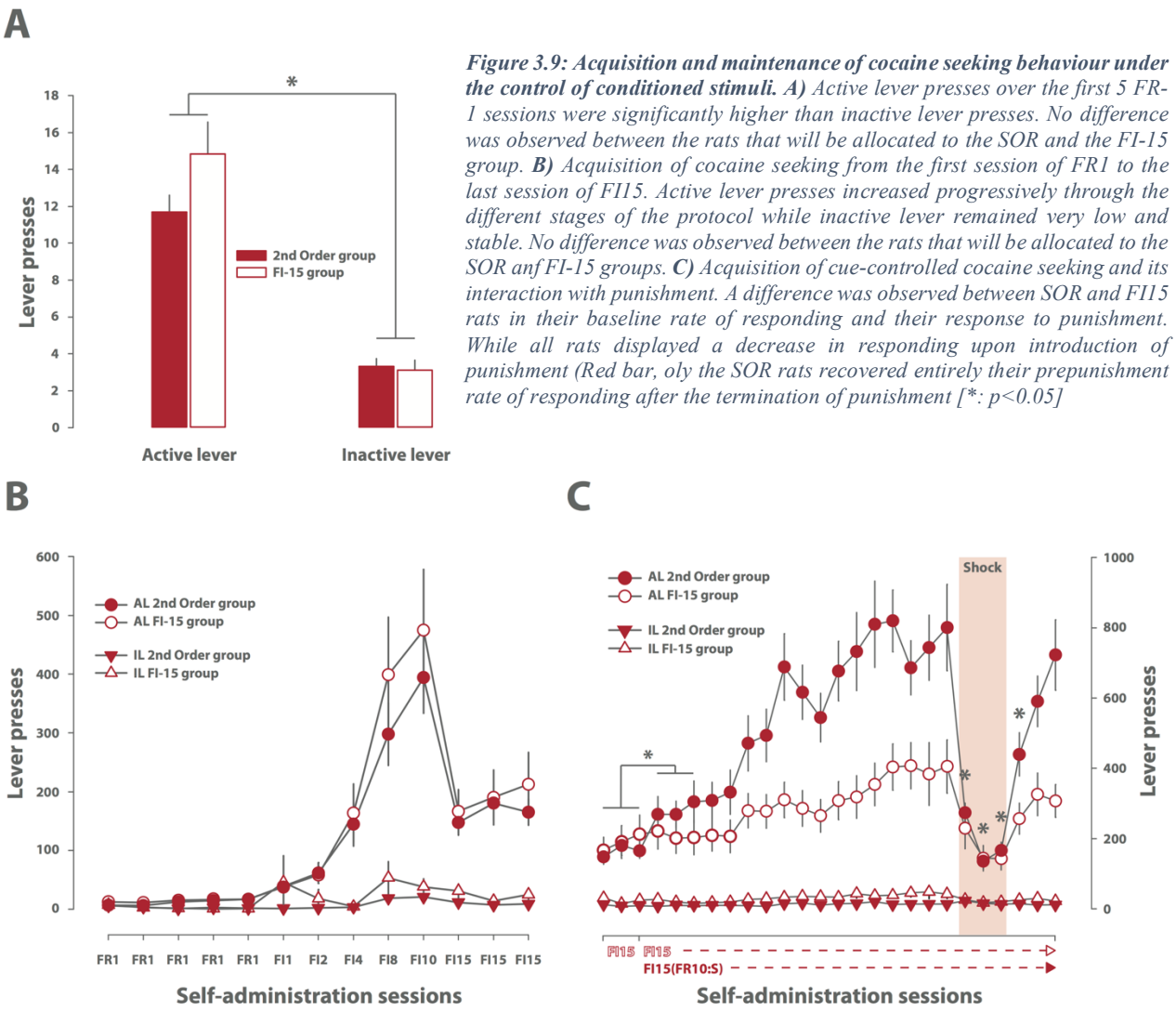


Figure 3.9: Acquisition and maintenance of cocaine seeking behaviour under the control of conditioned stimuli. A) *Active lever presses over the first 5 FR-1 sessions were significantly higher than inactive lever presses. No difference was observed between the rats that will be allocated to the SOR and the FI-15 group.* ***B)*** *Acquisition of cocaine seeking from the first session of FR1 to the last session of FI15. Active lever presses increased progressively through the different stages of the protocol while inactive lever remained very low and stable. No difference was observed between the rats that will be allocated to the SOR anf FI-15 groups.* ***C)*** *Acquisition of cue-controlled cocaine seeking and its interaction with punishment. A difference was observed between SOR and FI15 rats in their baseline rate of responding and their response to punishment. While all rats displayed a decrease in responding upon introduction of punishment (Red bar, oly the SOR rats recovered entirely their prepunishment rate of responding after the termination of punishment [*: p<0.05]*

Introduction of SOR in the other group resulted in an immediate and pronounced increase in active lever presses compared to the FI-15 sessions as shown in the first 3 SOR sessions *[main effect of group: $F(1,39) < 1$; bloc: $F(1,39) = 8.694$, $p < 0.05$; and group x bloc interaction $F(1,39) = 4.5386$, $p < 0.05$].* As previously described for heroin, this effect persisted over time *[main effect of session: $F(19,551) = 31.448$, $p < 0.0001$; lever: $F(1,29) = 56.821$, $p < 0.0001$; and session x lever interaction $F(19,551) = 30.649$, $p < 0.0001$; main effect of group: $F(1,39) = 4.4726$, $p < 0.05$; and session x group interaction: $F(16.624) = 3.9181$, $p < 0.0001$]* (**Fig. 3.9C**).

The introduction of contingent foot-shocks resulted in a marked decrease in active lever presses both in the FI15 and SOR groups *[main effect of group: $F(1,39) = 4.2426$, $p < 005$; session: $F(25,975) = 15.914$, $p < 0.0001$; and group x session interaction: $F(25,975) = 4.3967$, $p < 0.0001$]. Post-hoc analyses* confirmed that the number of active responses were significantly lower during shock sessions as compared to baseline only in the SOR group, and that they remained lower than pre-punishment baseline during the first post-shock session, as observed in the heroin experiment (**Fig. 3.9C**).

b. Characterisation of cocaine seeking during the first drug-free interval

During the first drug-free interval, Cs presentation resulted in an increase in instrumental responding in the SOR group *[main effect of session: $F(16,160) = 5.5189$, $p < 0.0001$]* (**Fig. 3.10A**). The average active lever presses displayed by the SOR group once the CS was introduced (red horizontal bar *(90.25 ± 13.10)*) was higher than both the mean of the three FI-15 sessions (grey horizontal bar *(21.65 ± 1.42)*) and the FI-15 group (red-framed grey horizontal bar *(48.01 ± 5.61)*) *[main effect of group: $F(1,39) = 4.1562$, $p < 0.05$; and group x session interaction: $F(16,624) = 2.0876$, $p < 0.01$]* (**Fig. 3.10A**).

Active lever presses during the first 15 minute interval of the 3 pre-shock sessions, the 3 shock sessions (red bar) and the 3 post-shock sessions are shown in **Fig. 3.10B**. Active lever presses dropped from baseline when shocks were introduced for the SOR group and remain lower than the baseline over post-shock block of sessions *[main effect of block: $F(2,58) = 4.9937$, $p < 0.05$]*. However, the level of responding displayed by FI-15 rats was bit significantly altered by punishment, as there was no difference between baseline, shock and post-shock sessions *[main*

effect of block: F(2,20) = 2.3697, p>0.1].

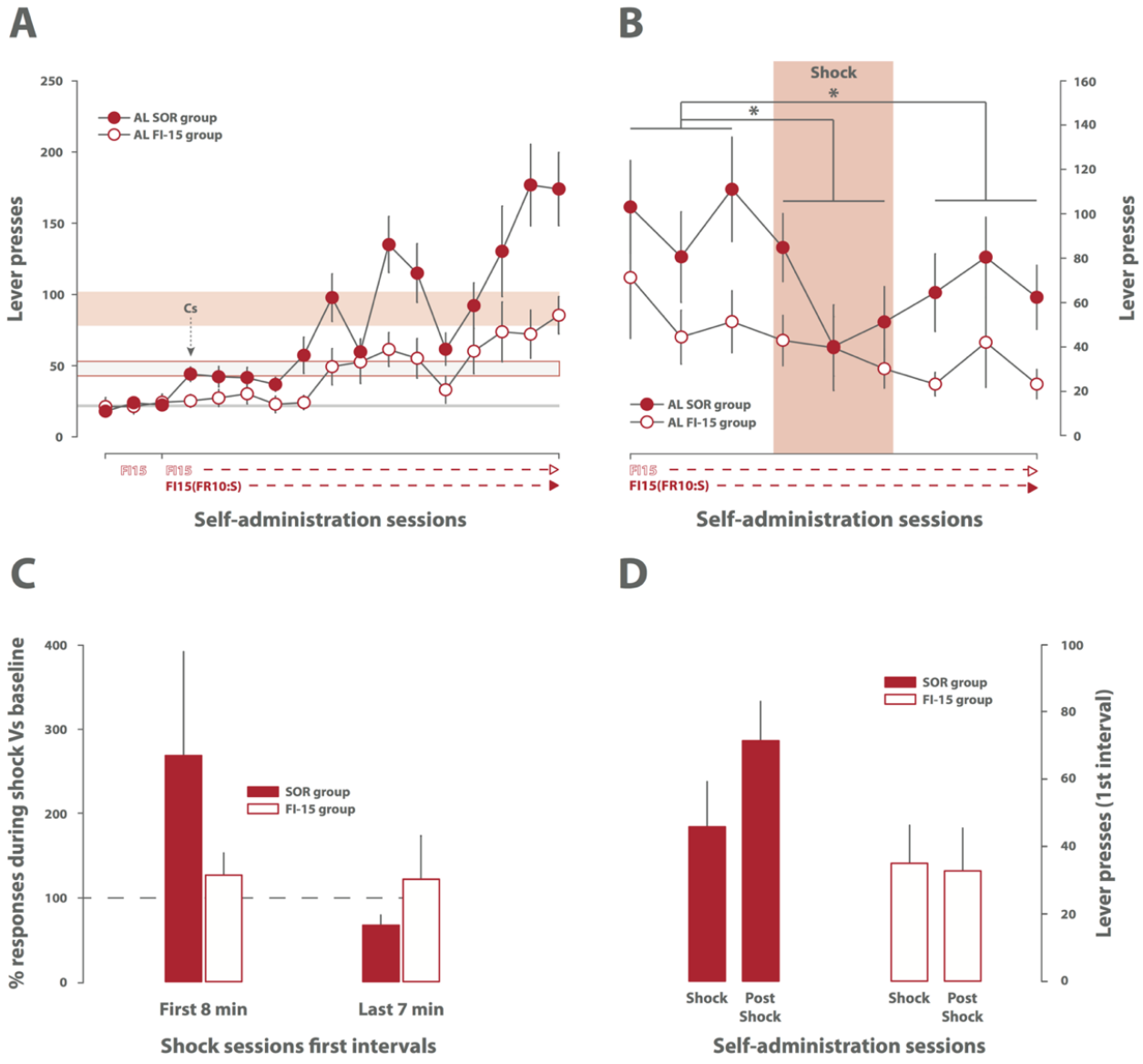


***Figure 3.10: Cocaine seeking behaviour displayed during the first drug-free 15min interval**. **A)** Active lever presses during the first interval increased progressively over time both in the FI15 and the SOR groups but were much higher in the SOR group after intrduction of the contingent presentation of the CS so that the average response rate under SOR (red horizontal bar) was higher than during the three FI15 sessions (grey horizontal bar) and those displayed by the FI15 group (grey red-framed horizontal bar). **B)** Active lever presses during punishment and, flancking baseline and post-shock sessions. The SOR group displayed a decrease in active lever presses upon introduction of punishment in contrast to the FI-15 group, whose level of responding was impervious to punishment. **C)** SOR rats displayed a trend twards an increase in their rate of responding during the first 8 min of the punished sessions as compared to baseline although this effect was not statistically significant. Shock presentation during the last 7 min resulted in a slight decrease (non statistically significant) in active lever presses compared to the pre-shock level of responding in the SOR group. D) SOR rats displayed a trend to respond more once the punisment was terminated (non statistically different) as compared to FI-15 rats which did not change their level of responding during and after punishment. [*: p<0.05]*

When considering the temporal distribution of the responses within the first interval, the level of active responding displayed by FI15 rats was insensitive to punishment across the 2 periods (unpunished and punished) *[main effect of period: F(1,10) < 1]*. However, SOR rats supressed drug seeking responses during the punished period of the first interval (below 100%), but displayed a trend toward increased responding during the first 8 minutes as compared to baseline (above 100%) but no significant difference was observed between the 2 periods of the interval *[main effect of period: F(1,29) = 3.1103, p>0.05]* (**Fig. 3.10C**).

Once punishment was terminated, the SOR group displayed a trend to increase their active responses over the post-shock sessions, in contrast, the FI-15 group maintained a similar level of responding to the one displayed during the three shock sessions. This increase, albeit only at trend level, was further supported by the comparison of the average AL presses during the last 2 post-shock sessions to the last 2 shock sessions *[main effect of group: F(1,39) = 1.8174, p>0.1 ; block: F(1,39) < 1; and group x block interaction: F(1,39) = 1.2673, p > 0.1]* (**Fig. 3.10D**).

3. From Habits to compulsivity.

The previous analyses were concerned with incentive habits and their contribution to resistance to, and recovery from, punishment as compared to "classical" habits, i.e. as measured under FI schedules of reinforcement. However, inter-individual differences were not taken into consideration. Therefore, the following set of analyses focused on inter-individual differences in compulsive drug seeking among the SOR population to better characterise compulsive heroin seeking and compare its features to compulsive cocaine seeking using the same procedure.

a. SOR heroin

A K-means cluster analysis, carried-out on the number of shocks received during the first interval of the last two shock sessions, segregated rats in three non-overlapping populations as follows: High compulsive rats (HC; n = 10), Intermediate compulsive rats (IC; n = 17) and Low compulsive rats (LC; n = 19) (**Fig. 3.11A-B**).

As expected from the nature of their selection, HC rats received more shocks than LC rats during the three 2-hours punishment sessions [*main effect of group: F(1,27) = 19.01, p < 0.001 : session: F(2,54) = 13.94, p < 0.0001; and session x group interaction: F(2,54) < 1*] (**Fig. 3.11C**).

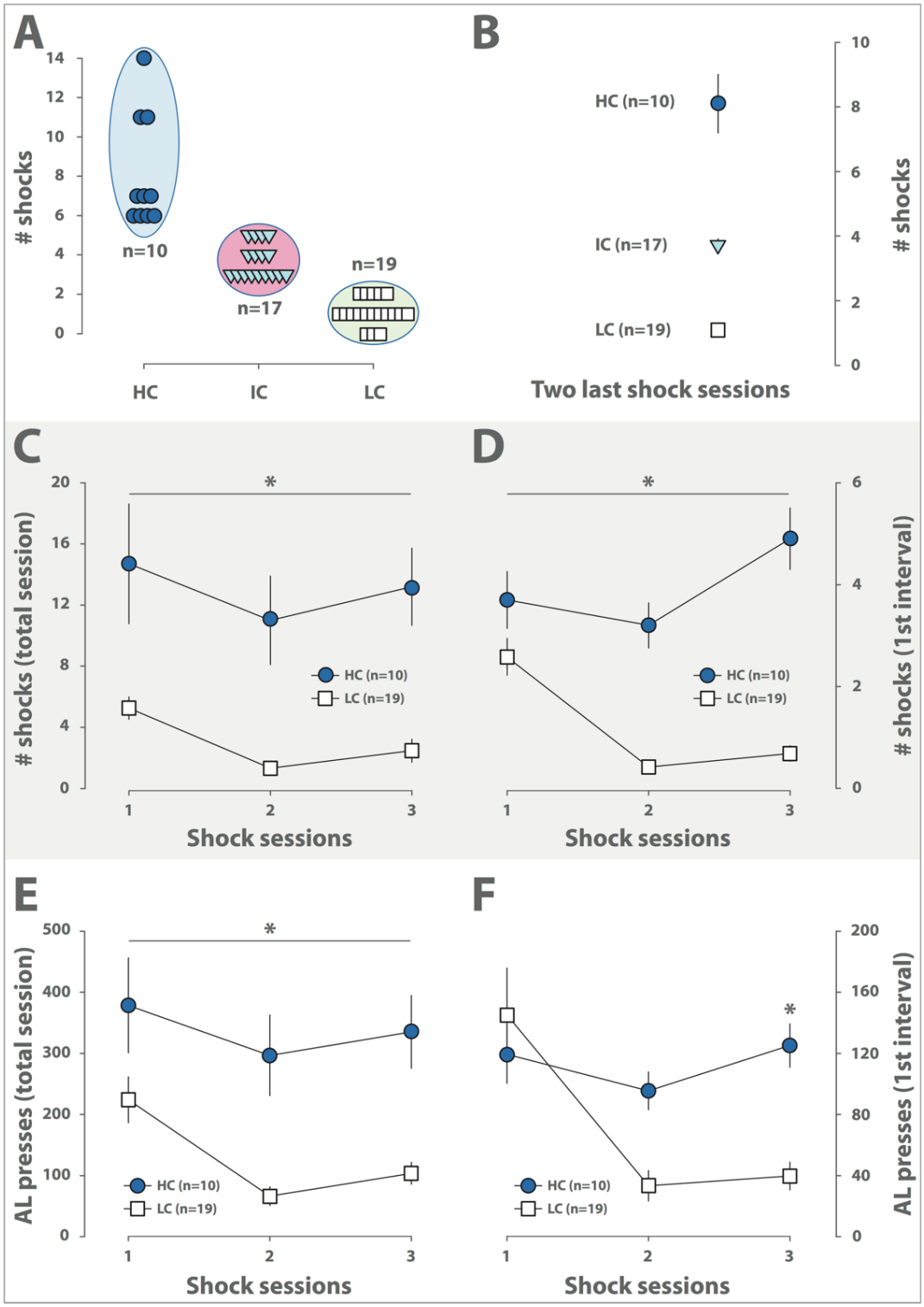


Figure 3.11: Identification of compulsive heroin seeking behaviour in rats displaying incentive habits for heroin. A-B) *Segregation of three populations of rats with regards to compulsivity by a cluster analysis based on the number of shocks received in the first 15 min intervals of the last two punishmed sessions. HC rats (n = 10) received more shocks than IC rats (n = 17) and LC rats (n = 19) (HC = 8.1 ± 0.9 ; IC = 3.7 ± 0.2 ; LC = 1.1 ± 0.1).* ***C-D)*** *Number of shocks received during the 2 hours sessions and the first 15 min interval of the three shock sessions, HC rats received more shocks than LC rats except during the first interval of the first punished session.* ***E-F)*** *Active lever presses during, respectively, the entire session and the first 15 min interval of the three shock sessions. HC rats displayed a higher rate of responding than LC rats during the last two punished sessions and responded significantly more during the first interval during the last punished session. [*: p < 0.05]*

Similarly, HC rats received more shocks during the first, drug-free, interval *[main effect of group: F(1,27) = 51.17, p < 0.0001 : session: F(2,54) = 10.83, p < 0.001 ; and session x group interaction: F(2,54) = 13.67, p < 0.0001]*. However there was no difference between groups during the first interval of the first shock session, demonstrating that LC rats progressively learnt to suppress their drug seeking responses (**Fig. 3.11D**), as previously reported for other drugs [200].

Similarly, HC rats displayed a higher rate of responding than LC rats over the 2-hour punished sessions [main effect of group: F(1,27) = 14.37, p < 0.001 : session: F(2,54) = 15.67, p < 0.0001 ; and session x group interaction: F(2,54) = 2.03, p>0.1] (**Fig. 3.11E**). However, no differences in active lever presses were observed during the first interval between until the last punished session. [main effect of group: F(1,27) = 3.86, p = 0.05989 : session: F(2,54) = 7.83, p < 0.01 ; and session x group interaction: F(2,54) = 5.46, p < 0.01] (**Fig. 3.11F**).

The difference in maintaining seeking behaviour despite negative consequences between HC and LC rats could not be attributable to a differential sensitivity to pain. Firstly, the resistance to punishment observed in HC rats during the first interval occur with no drug on board, and critically there was no difference between HC and LC rats in their pain threshold, as assessed by a hot-plate test prior to the punished sessions *[main effect of group: F(1,10) = 1.77, p>0.1]* (**Fig. 3.12**).

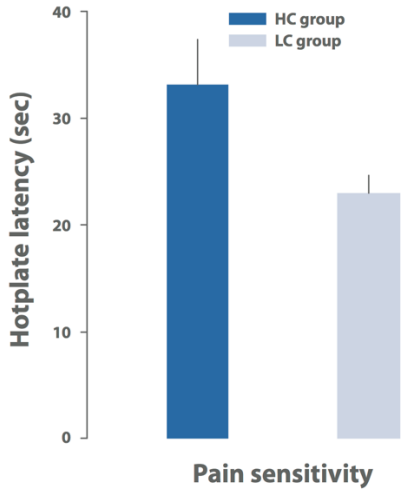


Figure 3.12: HC and LC did not differ in their sensitivity to pain. *Latency before apparition of pain-related behaviours on a hot-plate (52 celsius degrees). No significant differences were observed between HC and LC rats.*

b. SOR cocaine

Based on the number of shocks SOR cocaine rats received in the first interval of the last two shock sessions (which followed a tri-modal distribution, as shown in **Fig. 3.13**), two non-

overlapping subpopulations were segregated as follows (based on a median split as described in chapter 2): High Compulsive rats (HC; n = 6), and Low Compulsive rats (LC; n = 24) (**Fig. 3.14A**).

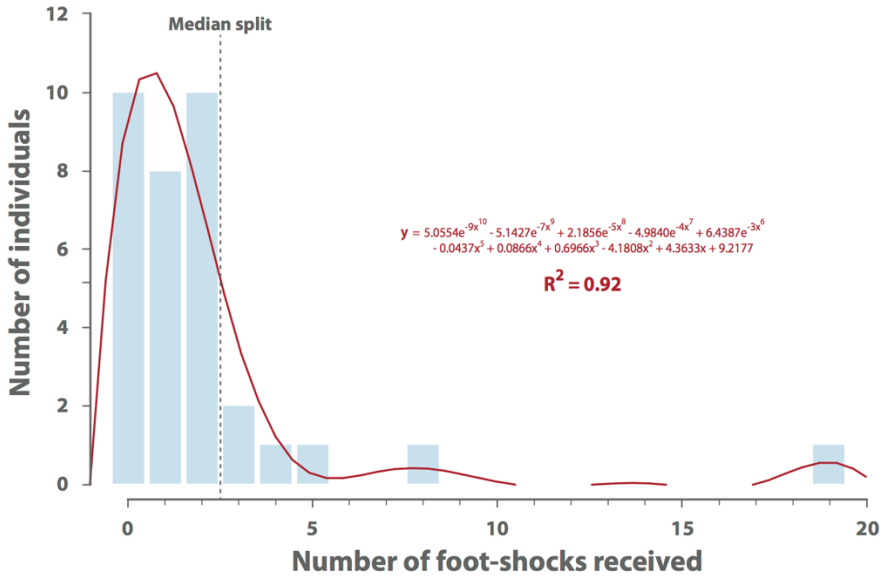


***Figure 3.13: Tri-modal distribution of the cocaine SOR population stratified on the number of shocks received during the first interval of the last two punished sessions**. The population displayed a tri-modal distribution, illustrated by the fitting curve plotted with a 10th order polynomial equation (red line). The median was calculated (med = 2) and the population was split in two groups: High compulsive rats, above median, receiving at least 3 shocks and Low compulsive rats receiving 2 or less shocks.*

As per the nature of their selection, HC rats received more shocks than LC rats over the 2-hour punished sessions [main effect of group: $F(1.28) = 2.1965$; $p < 0.05$, session: $F(2,56) = 55.033$, $p < 0.0001$; and session x group interaction: $F(2,56) = 4.3972$; $p < 0.05$] (**Fig. 3.14C**).

As observed for heroin, despite a main effect of group, post-hoc analyses revealed no difference between HC and LC rats in the number of shocks received in the first punished session [main effect of group: $F(1,28) = 15.148$, $p < 0.001$, session: $F(2,56) = 3.9238$, $p < 0.05$; and session x group interaction: $F(2,56) = 3.2595$, $p < 0.05$] (**Fig. 3.14D**).

HC rats displayed an overall higher rate of responding throughout the whole punished sessions, in comparison to LC rats *[main effect of group: $F(1,28) = 6.2494$, $p < 0.05$, session: $F(2,56) = 26.070$, $p < 0.0001$; and session x group interaction: $F(2,56) < 1$]*. Post-hoc analysis confirmed that the level of responding of HC rats was significantly higher than that of LC rats during the last two shock sessions (**Fig. 3.14E**). HC rats also displayed a higher level of responding than LC rats during the first interval of the punished sessions *[main effect of group: $F(1,28) = 10.782$, $p < 0.01$, session: $F(2,56) = 1.1632$, $p>0.1$; and session x group interaction: $F(2,56) = 3.9339$, $p < 0.05$]* (**Fig. 3.14F**).

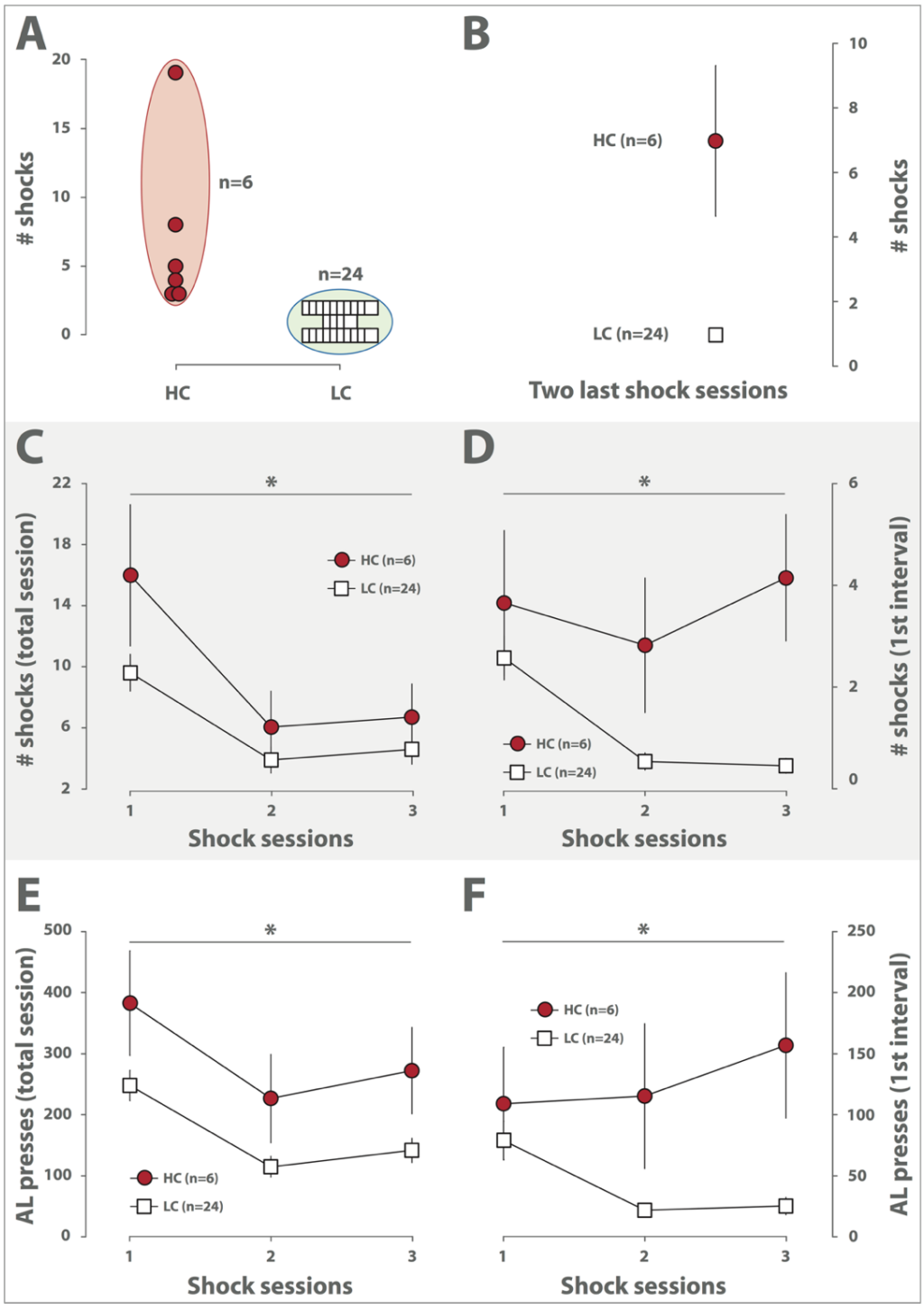


Figure 3.14: Identification of compulsive cocaine seeking behaviour in rats displaying incentive habits for cocaine. A-B) *Segregation of the two non-overlapping populations based on the number of shocks received during the first 15 min interval of the last two punishment sessions. High compulsive rats (HC, n = 6) received more shocks than low compulsive rats (LC = 24) (HC = 7 ± 2.5 ; LC = 1 ± 0.2).* ***C-D)*** *Number of shocks received during the 2 hour sessions and the first 15 min interval of the three shock sessions. HC rats received more shocks than LC rats over over the course of the punished sessions, both overall and during the first, drug-free interval.* ***E-F)*** *Active lever presses during the entire session and the first 15 min interval of the three shock sessions, respectively. HC rats displayed a higher level of responding than LC rats over the course of the punished sessions both overall and during the first drug-free interval. [*: $p < 0.05$]*

The difference in resistance to punishment observed between HC and LC was not related to a difference in pain sensitivity as they did not differ in their pain threshold; as assessed by a hot-plate test prior to the punished sessions *[main group effect: F(1,28) < 1]* (**Fig. 3.15**).

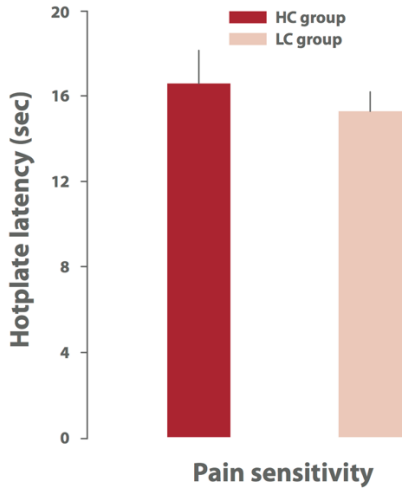


Figure 3.15: HC rats did not differ from LC rats in their pain thresholds. *Latency before apparition of pain-related behaviour on hot plate (52 celsius degrees). No significant differences were observed between HC and LC rats.*

Discussion

In this study, I first investigated the influence of incentive habits over the sensitivity to punishment and its recovery, and then characterised inter-individual differences in the development of compulsive cue-controlled heroin seeking.

All rats trained under a SOR acquired and maintained high levels of drug seeking behaviour under the control of conditioned stimuli. As compared to the performance of rats maintained under a FI15 schedule of reinforcement, which progressively engages S-R control over responding [257-259], the introduction of contingent presentations of heroin- (and cocaine-) paired CSs every tenth lever press resulted in a marked increase in drug seeking that persisted and even increased (or incubated, perhaps?) over several weeks. This invigoration reflects the magnitude and robustness of the control exerted by the reinforcing properties of CSs over drug seeking, an over exposure to which has been suggested to trigger the instantiation of incentive habits [145]. The qualitative and quantitative nature of conditioned reinforcement observed here for cocaine and heroin is in agreement with previous studies, although the overall rate of responding for heroin was higher in this study than previously reported [201, 260, 261]. This may be due to a difference in the strain of rats used, as this is the first evidence of well-established SOR for heroin

and cocaine in Sprague Dawley rats, previous experiments from the "Everitt's lab" having been performed on Lister Hooded.

Similarly, the aforementioned vertical drift in the level of responding under SOR is a classic feature of the behaviour of rats trained under this schedule, and, it also appears, albeit to a lesser extent, in rats trained under FI15. This 'drift' may reflect both the progressive establishment of automaticity for the latter and the development of incentive habits for the former, whereby the animals learn the reinforcing properties of the conditioned reinforcer. Clearly this needs further investigations, but current analysis of the time-course and the slope of the increase in drug seeking as predictive factors of transition to compulsivity have yielded no meaningful lead.

One promising avenue for future research is to investigate whether the devolvement of drug seeking to dopamine-dependent aDLS mechanisms, which is subject to major inter-individual differences [262], can be predicted by this increase in responding over time. Additionally, research has hitherto focused on the psychological and neural basis of drug seeking under SOR, such as the recent evidence that the dopamine-dependent intrastriatal functional shifts underlying the development of incentive habits, that are fully engaged after 15 to 20 days of training [146, 202], are under the control of functional transitions in the amygdala [146, 202, 245]. However, nothing is known about the neurobiological basis of over-trained performance under FI15 schedules of reinforcement.

The habitual nature of seeking responses is commonly probed behaviourally by measuring sensitivity to devaluation of the outcome or degradation of the contingency [236, 263]. Alternatively, habitual responding can be probed by measuring the sensitivity of performance to causal manipulations of the aDLS [239, 264]. Outcome devaluation has been widely used and is almost exclusively effective when paired with oral reinforcers. Devaluation of the motivational properties of intravenously self-infused reinforcers has proven almost impossible because this route is dissociated from any post-ingestive incentive learning mechanisms by which outcome devaluation is mediated [239], but see [265, 266].

One of the potential avenues to parametrically reconcile the neural and behavioural assessment of habitual control over heroin and cocaine seeking, as measured here, under either FI15 or SOR would be to physically dissociate the seeking from the taking manipulandum by

adapting the seeking-taking task to the FI schedules of reinforcement whereby once each interval has elapsed a response on the seeking lever would give access to a taking lever as used by Zapata and colleagues [239]. Such a procedure, which has been recently developed in the Belin lab by Dr. Aude Belin-Rauscent will offer unique opportunities to investigate the extent to which resistance to devaluation of the outcome of the seeking response predicts resistance to punishment, and offer a definitive conclusion as to the relationship between habits and compulsivity.

Indeed, the maintenance of drug seeking despite contingent presentation of aversive foot-shocks in a heroin-free state, as measured during the first 15 min interval of SOR sessions is the best operationalisation of the compulsive nature of heroin seeking displayed by individuals who are addicted to the drug.

Punishment as introduced in our procedure, had the same influence on heroin seeking behaviour in both SOR and FI-15 groups, namely it markedly decreased responding over the three sessions during which it was applied. This suggests that neither habits nor incentive habits (as defined as the sensitivity of the response to dopamine receptor blockade in the aDLS) contribute to compulsivity, at least if it is defined as resistance to contingent punishment of the response. However, incentive habits were shown here to specifically facilitate the expression of maladaptive drug seeking behaviour in anticipation of, and following, punishment. Thus, rats trained under SOR increased their level of seeking responses during the first 8 minutes of punished sessions as compared to baseline sessions. Thus, I would argue that the SOR rats, whose instrumental responses are governed by incentive habit mechanisms, anticipate the inability to respond due to negative consequences during the punished period of the interval, and demonstrate an aberrant drive to express the response at a time when it is not appropriate, i.e. in the first 8 minutes. I further argue that this adaptation could be considered aberrant as instrumental responding during the first 8 minutes is temporally remote from drug infusion, which rats learnt over several weeks. Thus, in the face of a future loss of opportunity to respond freely, rats showing incentive habits engage in aberrant responding beforehand, as well as once punishment is terminated, since only SOR rats displayed a recovery of their pre-punishment seeking responses then. This maladaptive behavioural adaptation to punishment, whereby rats

displaying incentive habits exhibit a drive to express their drug seeking response when environmental conditions hamper the natural expression of their habit (as predicted by the psychological model of incentive habits, see **Fig. 3.3**), challenge the way compulsivity has been initially operationalised by Belin and colleagues [193]. In addicted individuals, and in this study in rats displaying incentive habits, the compulsive nature of drug seeking is seldom manifested while negative consequences are experienced contingently. Instead, individuals addicted to drugs engage in aberrant drug seeking behaviour despite the knowledge of future negative consequences, or despite having endured negative consequences in the past. For instance, individuals addicted to heroin who just spent some time in jail because of a crime they committed related to procuring the substance, may resume their drug seeking behaviour as soon as they leave prison, but they do not actually face the adverse consequences of doing so at that time. They simply cannot take into account the fact that they had just been exposed to adverse consequences. Similarly, compulsivity in rats may not only refer to the resistance to actual contingent punishment, but also, and perhaps most critically, to the nature of the behavioural adaptations in anticipation of, and following, punishment.

Interestingly, these aberrant behavioural responses could be somehow related to the emergence of drug seeking in rats characterised as “3 criteria” in the model developed by Belin and colleagues [174, 192, 193] during the periods of self-administration sessions in which the drug is not available and signalled as such. This increase in responding, that only occurs after protracted exposure to the drug, considered to reflect an inability to withhold drug seeking responses in compulsive rats, may reflect a similar psychological construct as the one observed in this study.

These behavioural adaptations to punishment specifically displayed by rats exposed to SOR for heroin, were also displayed by those exposed to SOR, but not a FI15 schedule of reinforcement for cocaine, albeit to a more marginal degree.

The fact that we reproduced the observed effect with cocaine demonstrates the robustness of the phenomenon and offers further support to the importance of incentive habits in maladaptive drug seeking behaviour across several classes of drugs. However, the lack of statistically significant effects in the case of cocaine warrants further discussion. Several factors

may have contributed to this lack of statistical power. Firstly, the size of the population was smaller in the SOR cocaine group (n = 30) as compared to SOR heroin group (n = 46) and could potentially explain the lack of statistical power due to the high inter-individual differences in the level of responding observed in the face of the punishment. Secondly, the SOR schedule of reinforcement for heroin has been suggested to promote the functional recruitment of aDLS control over behaviour faster than for cocaine (unpublished data) . Consequently, while incentive habits may have been fully developed in rats seeking heroin, when punishment was introduced, from the 18th session onwards, potentially some rats had not yet fully developed incentive habits for cocaine.

Also, under the schedule of punishment used, the occurrence of shocks is linearly linked to the response rate which resulted in a higher number of shock received by SOR heroin rats as compared to SOR cocaine rats. The higher number of shocks received by SOR for heroin rats could also be explained by an overall lower sensitivity to pain as compared to those exposed to SOR for cocaine measured at the population level in the hot-plate test. This pain threshold assessment was always carried-out 6 hours following the behavioural session in order to avoid any interference with the acute analgesic effect of heroin. As a population, rats trained under SOR for heroin displayed a lower sensitivity to pain than those trained for cocaine, suggesting that chronic self-administration of heroin may induce long-term adaptations within the pain system which, while not accounting for the inter-individual differences in the vulnerability to develop compulsivity, may explain the differential level of exposure to shocks, hence the level of compulsivity, between the two groups.

Considering the contingency we introduced between responding and punishment, the probability of receiving shocks was baseline response dependent. Therefore, rats trained under SOR were more likely to receive more punishments early, in comparison to those trained under an FI15 schedule of reinforcement. If one wanted to further compare these two schedules of reinforcement, this potential bias could be circumvented by delivering shocks non-contingently, according to a variable schedule (i.e. FI-15[(FR10:S)/VI-90(FR5)]). The punishment would still occur within the last 7 minutes of each 15 min interval but would follow a 90 seconds variable interval schedule after completion of 5 active lever presses so that rats unwilling to respond in

the face of punishment would not receive further shocks.

Since incentive habits, previously shown to be underlined by aDLS dopamine-dependant mechanisms facilitate an aberrant, compulsive, engagement of behaviour, they offer new insight into the understanding of the complex psychological mechanisms that lead to compulsive behaviour. Therefore, I decided further to investigate the compulsive nature of heroin and cocaine seeking only in rats expressing incentive habits.

The breadth of individual variability in the propensity to maintain drug seeking in the face of punishment enabled the identification of several non-overlapping compulsive phenotypes based on the number of shocks received in the first interval of the last 2 punishment sessions within the populations of rats trained under SOR for heroin and cocaine. The size of the population in the heroin experiment (n = 46) was sufficient to be subjected to an unbiased segregation by cluster analysis in three non-overlapping subpopulations while this was not possible for the cocaine experiment. The size of cocaine SOR group was expected to be large enough to assess compulsivity levels but the low level of responding previously discussed, and the exclusion of six subjects due to loss of catheter patency not only narrowed the population but also prevented its discrimination into three subpopulations. However, splitting the population above the median to obtain two subpopulations lead to the identification of a reasonably similar percentage of rats displaying a high compulsive profile (20%) as compared as those described in the heroin experiment (21.74%), thereby supporting the differential strategies deployed to assess levels of compulsivity in these two independent experiments. Using the seeking-taking task, Pelloux and colleagues [197] showed that, in a population of 21 rats with a history of extended access to cocaine, 5 of them (or 23.8%) showed resistance to punishment. This percentage is remarkably close to the one represented by the HC population in the present study.

Further behavioural characterisation of the nature of the compulsive phenotype displayed by the rats shown here to be vulnerable would offer new insights in our understanding of heroin addiction and the factors that contribute to this vulnerability. For instance, as previously discussed, whether compulsivity is preceded, and predicted by resistance to devaluation of the outcome of the seeking response, or whether it is best predicted by the motivation for the drug (a hypothesis slightly at odds with the fact that the behaviour is likely under S-R control at that

stage, but still important to test) and if it predicts increased propensity to relapse, as measured under extinction, after abstinence.

Nevertheless, having established a behavioural procedure whereby individuals vulnerable to develop compulsive heroin seeking behaviour could be identified within a relative short period of time, I was poised to investigate the behavioural factors of vulnerability to develop compulsive heroin seeking habits.

CHAPTER 4: IDENTIFICATION OF BEHAVIOURAL MARKERS OF VULNERABILITY TO COMPULSIVE HEROIN SEEKING BEHAVIOUR

Introduction

The investigation of behavioural markers of vulnerability to develop compulsive drug seeking relies on the assumption, supported by wealth of evidence, that rats, like humans, display a distinct collection of behavioural traits. Moreover, such traits are manifested both in ethological conditions and within specifically-designed tasks which offer an easier quantification in standardised conditions.

A strong association between various personality traits including anxiety, sensation seeking or impulsivity and psychiatric disorders such as drug addiction in humans has been repeatedly demonstrated [91, 92, 99] and extensively reviewed in the context of addiction [138, 267-269]. Such traits have been shown to be associated with behavioural features of addiction to psychostimulants [270, 271], alcohol [272], opiates [72, 273] and tobacco [274]. However, it is almost impossible to determine in clinical studies whether these traits pre-date drug use, thereby representing endophenotypes of vulnerability, or if they are simply a consequence of drug exposure, irrespective of the development of an addiction. One way of addressing this crucial issue is to implement longitudinal studies in rats in which behavioural traits are characterised in large cohorts of outbred individuals prior to exposing animals to drugs and identify those that eventually develop the behavioural features of compulsive drug seeking and taking.

Thus, preclinical studies based on animal models have successfully identified a number of behavioural traits that predict an increased propensity to engage in drug use, to respond to the incentive properties of the drug or to develop compulsive drug intake. These behavioural traits also have relevance with regards to comorbid elements of individuals addicted to drugs, as they relate to anxiety, sensation seeking, individual vulnerability in attribution of incentive salience (sign tracking), decision making or sensitivity to natural rewards (referring to general hedonic states).

1. Anxiety

Drug use is acquired as a goal-directed behaviour whereby individuals may initially experiment with drugs for various reasons including peer-pressure or to cope with lack of self-esteem, shyness or affective distress (self-medication) as exhibited by individuals showing high levels of anxiety [275]. Anxiety-like behaviours are usually operationalised in rodents by challenging their natural fear of bright and open spaces. Therefore, a greater number of the entries into, and time spent in the open arms of an EPM under mild stress conditions (described in chapter 2) represents a reliable marker of low anxiety trait, as compared to rats preferring the "protected" closed arms [206].

The construct validity of the EPM has been further validated through pharmacological isomorphism, in that performance (albeit in different experimental conditions to those used in the present study, including the light intensity) is linearly influenced by administration of either anxiogenic or anxiolytic drugs [207] with relatively good predictive validity with regard to the anxiolytic properties of those drugs in humans.

High anxious (HA) rats, as identified on the EPM, have been shown to display higher motivation for cocaine than their low anxious counterparts (LA) [276]. HA rats also display an increased propensity to develop high levels of CPP [277] and to prefer alcohol in comparison to LA rats [278]. HA rats were also shown to be more vulnerable than LA rats to escalate their intake of cocaine, but not heroin under extended access conditions [209], thereby suggesting that this behavioural trait does not interact the same way with psychostimulants and opiates.

2. Sensation seeking

Sensation seeking initially defined by Zuckerman [279] and further characterised by the factorial analysis of the sensation seeking scale carried-out by Arnett [279] has been associated with drug use and addiction [93-95]. This multifaceted behavioural trait is characterised by two independent factors, namely thrill seeking and novelty seeking. Thus individuals who score highly on that scale tend to be easily bored (boredom susceptibility, a component of novelty seeking) and seek novel, varied and complex environments and sensations (which refers to thrill seeking) [280]. High sensation seekers have also been shown to be more prone to engage in drug use [281,

282].

In rats the two sub-components of sensation seeking have been suggested to be operationalised by two distinct behaviours. Locomotor reactivity to novelty in an open field (OF) [283], has been used as a measure of thrill seeking, and the propensity to freely explore novel environments, as measured in a novelty-induced place preference (NPP) in CPP boxes [284], has been suggested to represent boredom susceptibility or novelty seeking, as described in chapter 2. In the former task, rats displaying higher locomotor reactivity to novelty (high responders, HR rats) have been shown to more readily acquire cocaine [192, 285] and amphetamine [286] self-administration as compared to low responder (LR) littermates. HR rats also have an enhanced AcbC dopamine release in response to cocaine [285, 287] and are more sensitive to the pharmacological effects of psychostimulants [286, 288, 289] than LR rats. Interestingly, Vanhille and colleagues [210] showed, using a mutually exclusive choice procedure, that despite their propensity to self-administer cocaine, HR rats were very sensitive to an alternative reinforcer, namely saccharine and gave up the drug for the sweetener. These data would suggest that HR rats are not more "addicted" to cocaine than LR rats. This is in line with the repeated finding by Belin and colleagues that HR rats are resilient to the transition from controlled to compulsive cocaine intake [192, 212].

In marked contrast, High novelty preference rats (HNP) characterised by their preference for the novel environment in the NPP task differ from low novelty preferred (LNP) animals in their expression of CPP for amphetamine [290], but not in their propensity to acquire self-administration for this drug [291]. Belin and colleagues [212] further showed that HNP rats displayed a greater tendency to develop a multi-symptomatic addiction-like behaviour for cocaine than LNP rats. These data further demonstrate that the two behavioural assessments of sensation seeking in rats are independent and predictive of distinct stages of drug use history, namely the acquisition of drug use for the HR trait and the transition to addiction for the NHP trait [290, 292].

3. Impulsivity

Alongside novelty preference, high waiting impulsivity (as preclinically measured in the 5-

choice serial reaction time task [267, 293-295]) has been shown to be an endophenotype of vulnerability to develop compulsive cocaine self-administration in rats [192] and addiction in humans [296]. High impulsivity trait in rats predicts an increased propensity to escalate cocaine self-administration, and is associated with a decrease in $D_{2/3}$ dopamine receptor binding and alterations of GABAergic markers in the nucleus accumbens [297]. High impulsive rats also show an increased propensity to develop compulsive-adjunctive behaviours as compared to low impulsive rats, but do not display an increased vulnerability to escalate heroin self-administration [189], suggesting that impulsivity may not necessarily contribute to the vulnerability to opiate addiction the way it has been shown for stimulant addiction. For this reason, and also because of time and experimental constrains (the identification of HI rats requires at least 70 daily sessions, and it is not compatible with the measurement of decision making, as both task utilise the same set-up) the contribution of high impulsivity trait to the vulnerability to develop compulsive heroin seeking behaviour has not been investigated in this study.

4. Aberrant attribution of Incentive salience (sign tracking)

The incentive-sensitisation theory, developed by Robinson and Berridge [140], suggests that repeated exposure to addictive drugs results in the sensitisation of the dopamine-mediated reward pathway in the brain. This sensitisation results in an augmented response to the incentive and associative properties of the drugs, divorced from their subjective effects (so-called liking), that lead behaviourally to an aberrant motivation for the drug as subjectively expressed during craving. This theory, which relies heavily on an aberrant engagement in associative learning mechanisms by addictive drugs, postulates that neutral environmental stimuli acquire 'an aberrant incentive motivational salience' when repeatedly paired with drug administration, through a hijacking of Pavlovian conditioning mechanisms by these drugs [140]. Therefore, these previous neutral stimuli become CSs, sufficient to cause relapse and craving in human drug addicts [298] and also contribute to the maintenance of drug use and precipitate reinstatement of instrumental responding following abstinence or extinction in animals [201, 299].

However, there are marked inter-individual differences in the tendency to ascribe incentive value to conditioned stimuli, which may contribute to the vulnerability to addition [300]. There is a marked dissociation in conditioned approach to the magazine (spatially proximal to

reinforcement) or the conditioned cue (predictive of reinforcement, spatially distant) upon CS presentation shown by subpopulations of rats exposed for a limited time to an autoshaping procedure [203, 300]. Rats characterised as sign-trackers (ST) display increased approaches toward the CS, whereas rats that show more approaches to the locus of the reward delivery are characterised as goal-trackers (GT). As compared to GT, ST rats utilise phasic dopamine transmission for stimulus-reward incentive associations [23]. ST rats also display higher motivation for cocaine self-administration and show higher propensity to cocaine-induced reinstatement [301] in comparison to GT rats.

5. Decision making

Decision making deficits have been associated with several impulse control disorders, including drug addiction [81, 82, 84, 85, 302] and obsessive/compulsive disorders [303]. Decision making deficits have broadly been considered to arise as a consequence of neuropsychiatric disorders, especially following exposure to addictive drugs. However, these deficits may represent a pre-existing trait that facilitates the emergence of impulsive / compulsive disorders in vulnerable individuals. Decision making under uncertainty is assessed in humans by the Iowa Gambling Task (IGT) developed by Bechara and colleagues [304]. This task simulates real-life decision making and reveals marked inter-individual differences in the ability to maximise gains [305]. A rodent analogue of the IGT has been developed in rats, enabling the assessment of decision making prior to drug exposure. As described in chapter 2, and similar to the human version of the task, there are marked inter-individual differences in the ability to maximise reward in the rat version of this task (RGT). Rats that progressively prefer the advantageous options in the task are deemed good decision makers (GDM) whereas those that predominantly select from the high incentive/high loss holes are considered bad decision makers (BDM) [204].

6. Sensitivity to natural reward

Clear relationships have been reported in humans between sweet preference and abuse of opiates [306], cocaine [307] and alcohol [308]. Similarly, healthy individuals with a history of paternal alcoholism [309] display a high propensity to like sugar. Thus, it has been suggested that the mesolimbic dopaminergic pathway mediates a component of the hedonic responses to both

drugs and natural reward such as sucrose, indicating a similar underlying neurobiological mechanism for sugar preference and the pleasurable subjective feeling produced by some drugs of abuse [309].

In preclinical research, it has also been shown that sweetness preference is a marker of vulnerability to the reinforcing properties of addictive drugs. Rats bred for sweetness preference (saccharin preferring (HP)) have been shown to more readily acquire morphine [310], cocaine [311] and amphetamine [312] self-administration in comparison to rats displaying low levels of saccharine consumption (LP). To determine sweet preference in rodents, a sweet solution and water are presented in a two-bottles choice paradigm. Saccharin is often preferred over sucrose as the experimental sweet reinforcer, due to its sweet taste and its lack of calorific value which is often a confounding factor.

These behavioural traits, when measured prior to drug self-administration, have been mostly used in the context of psychostimulant use but few studies have been interested in the potential predictive nature of these traits to opiates addiction.

More importantly, most of the studies previously mentioned focused almost exclusively on drug reinforcement, motivation or reinstatement but did not investigate the contribution of these traits to the vulnerability to develop compulsive drug seeking habits.

In this study, rats were screened in these behavioural tasks prior to be subjected to heroin self-administration using the model described in the previous Chapter to probe whether these traits could be predictive of differential levels of acquisition of heroin self-administration, and of differential habitual and compulsive heroin seeking behaviours.

Materials and methods

1. General experimental design

Male Sprague Dawley rats (n = 47) in two independent cohorts were initially screened for the aforementioned behavioural traits prior to being exposed to heroin self-administration and heroin seeking under a SOR, after which they were identified as having developed compulsive heroin seeking behaviour or not.

In a first experiment, 23 rats were tested for anxiety-related behaviours, novelty-induced place preference and locomotor reactivity to novelty in the EPM, CPP boxes and the OF respectively (as described in chapter 2). In a second experiment 24 rats were tested for their incentive salience attribution to CSs, decision making and sensitivity to natural reward in the autoshaping task, RGT and two-bottles choice of saccharine versus water, respectively (as described in chapter 2) (**Fig. 4.1**). All rats subsequently underwent IV surgery as described in chapter 2. Following a week of recovery, rats were trained to self-administer heroin (0.04mg/infusion) in 12 operant chambers, as previously described.

Rats were trained to self-administer heroin under a FR1 schedule of reinforcement for 5 consecutive days, then under FI schedules of increasing duration (FI-1 to FI-15) for 8 daily sessions. Following these sessions, rats were maintained under a SOR schedule of reinforcement for 17 daily sessions. Subsequently, one part of each drug-free seeking period was punished by mild foot-shocks for three sessions as described in Chapter 3. Lastly, rats were then re-exposed to three SOR baseline sessions to investigate their ability to recover their pre-punishment drug seeking behaviour (**Fig. 4.1**). One rat from experiment 1 was excluded due to loss of catheter patency.

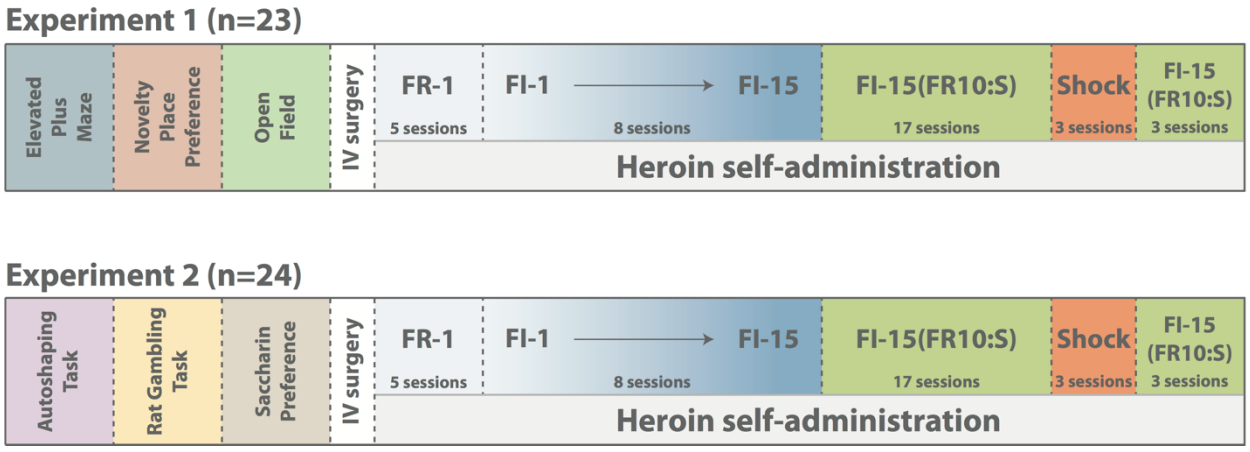


Figure 4.1: Schematic representation of the experimental design. *In a first experiment, 23 rats were screeened for their anxiety, novelty preference and locomotor reactivity to novelty. In a second experiment, 24 rats were screened for sign tracking, decision making and sesnsitivity to natural reward. Al rats were then subjected to IV surgery and subsequently trained to seek heroin under a SOR until they were tested for their resistance to punishment, as described in Chapter 3.*

2. Data and statistical analyses

a. Characterisation of behavioural traits

For all the behavioural traits, rats in each population were stratified and divided into subpopulations based on the parameters shown in **Table 4.1**. Prior to conducting any parametric statistical analysis of the datasets, the normality of the distribution of each group was assessed using the Sapiro-Wilk's test.

Following each behavioural task, the upper and lower quartiles of the population (n = 6 each) were identified as two distinct groups (as described in chapter 2), for which the nomenclature is shown below (**Table 4.1**).

Tests	Parametric parameters	Groups nomenclature
EPM	% time spent in the open arms over the 5 minutes test	**HA** High anxious **LA** Low anxious
NPP	% time spent in the novel compartment over time spent in both compartment	**HNP** High novelty preference **LNP** Low novelty preference
OF	Distance travelled over the 2 hours test	**HR** High responders **LR** Low responders
Autoshaping task	Average of lever presses displayed over the last 3 sessions	**ST** Sign trackers **GT** Goal trackers
RGT	% of good choices over the total trials	**GDM** Good decision makers **BDM** Bad decision makers
Sacc preference	% of saccharin intake over the total fluid intake (Box-Cox transformed)	**HP** High preference **LP** Low preference

Table 4.1: behavioural variables used to measure behavioural traits in the different tasks and associated nomenclature of the phenotype for each test.

The variable used to assess saccharin preference (i.e. percentage of saccharin intake over the total fluid intake) was found to be non-normally distributed due to the high amount of saccharin drank by the overall population as compared to water. Common linear transformations used in behavioural neuroscience (i.e. Log, square root, exponential, 1/x) failed to circumvent the non-normal distribution. Thus, a Box-Cox transformation which is a non-linear transformation, frequently used to transform distributions skewed toward 0 or a natural limit (in this case 100%), was applied [313]. This transformed distribution was very conservative and passed the Sapiro-Wilk's test, therefore, enabling further parametric analysis.

As described in chapter 2, anxiety-related behaviours in the EPM can be characterised by

different parameters, namely, entries or time spent in the open arms and head dipping in several compartments of the open arms (i.e. proximal, distal and terminal). Here, rats were characterised as either high (HA) or low anxious (LA) based on the percentage of time they spent in the open arms over the 5-min test.

b. Compulsivity

Compulsive heroin seeking was, as described in chapter 3, based on the number of shocks each individual was willing to receive in order to continue seeking heroin during the first interval of the last two punished sessions. The size of each population was too small to enable their segregation into 3 non-overlapping populations (namely HC, IC and LC) by k-means cluster analysis. We therefore, considered a percentage of HC rats that matches the one identified on a larger population as characterised in Chapter 3, i.e. the highest 20% of the population stratified on the resistance to shock. Maladaptive behavioural adaptations in the face of punishment, namely aberrant increases in responding during the first 8 min of the first punished intervals and recovery of pre-punishment levels of seeking during post-punishment sessions were also analysed, as described in Chapter 3.

Results

1. Characterisation of behavioural traits

In both experiments, the variables used to characterise the behavioural traits were normally distributed, as confirmed by the Sapiro-Wilk's test, and were therefore subjected to parametric analyses (**Fig. 4.2** and **Fig. 4.3**). The Gaussian curves displayed in red on each distribution represent the "expected" normal distribution. Each behavioural test enable the segregation of two distinct, statistically different, group of individuals (**Fig. 4.2** and **Fig. 4.3**).

In experiment 1, LA rats spent a greater amount of time exploring the open arms (OA) in the EPM as compared to HA rats *[main effect of group: $F(1,10) = 99.441$, $p < 0.0001$]* (**Fig. 4.2A**). HNP rats spent more time exploring a novel compartment that LNP rats *[main effect of group: $F(1,10) = 22.956$, $p < 0.001$]* (**Fig. 4.2 B**). HR rats displayed a higher locomotor activity throughout the 2 hours session than LR rats *[main effect of group: $F(1,10) = 57.381$, $p < 0.0001$; time: $F(11,110) =$*

26.682, p < 0.0001; and group x time interaction: F(11,110) = 2.9551, p < 0.01] (**Fig. 4.2 C**).

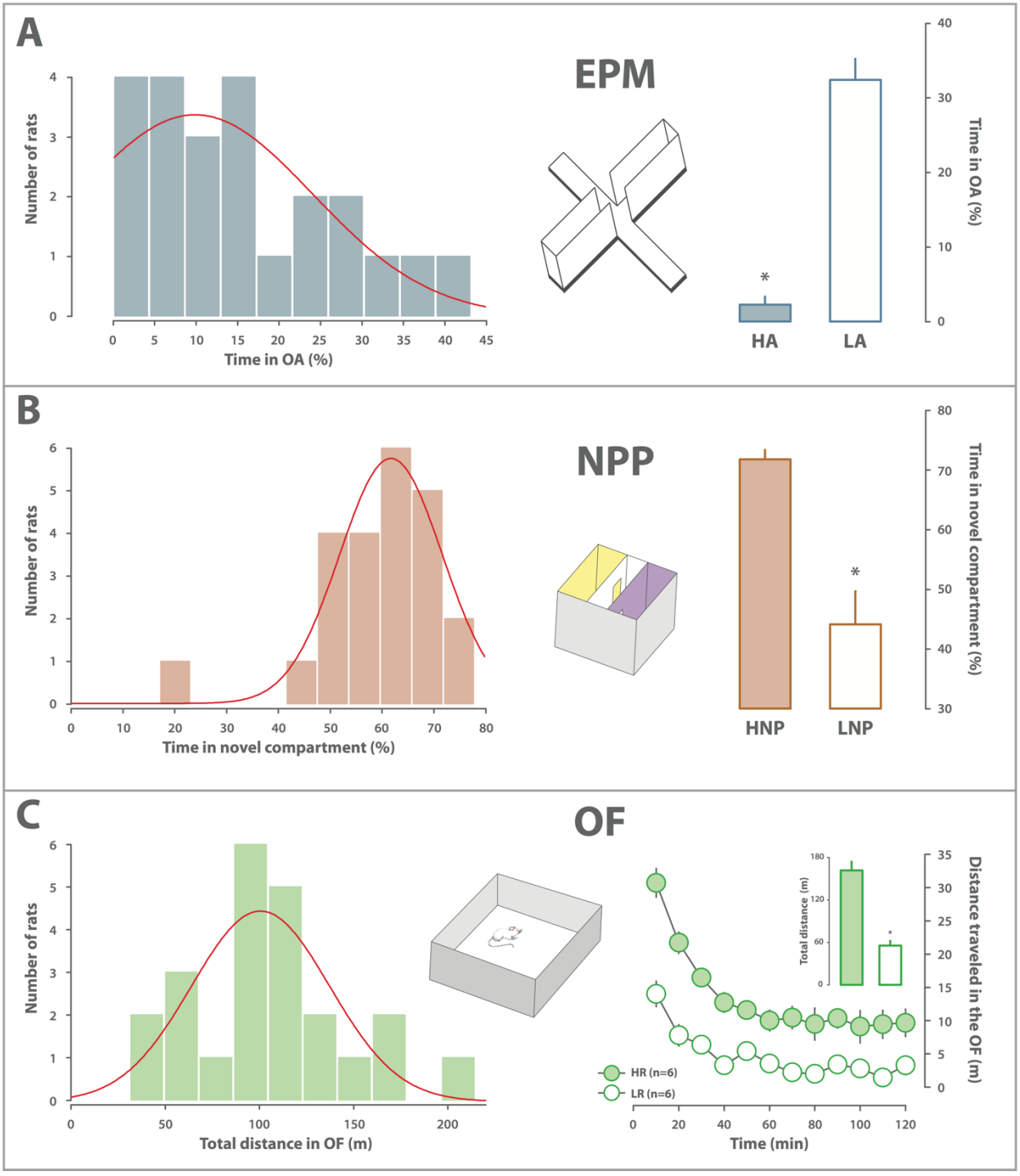


Figure 4.2: Behavioural traits assessed in experiment 1: Anxiety-related behaviour / novelty-induced place preference / locomotor reactivity to novelty. A) *LA rats displayed a higher percentage of time spent in the OA than HA rats.* ***B)*** *HNP rats displayed a higher percentage of time spent in a novel compartment as compared to LNP rats.* ***C)*** *HR rats displayed a higher locomotor activity as compared to HA rats. The distribution of the population for each trait with regards to the segregative variable was following a normal distribution (red curves show the expected normal distributions).[*: p< 0.05]*

In the EPM, the percentage of time spent in the open arms was positively correlated with the number of head dips in all the sub-territories of the open arms (i.e. proximal, distal and terminal) as shown in **Table 4.2**. LA rats, characterised based upon this criterion, displayed a higher occurrence of head dipping in all the compartments as compared to HA rats (**Table 4.2**).

categorical factors	One-way ANOVAs							
	Total head dipping		Proximal head dipping		Distal head dipping		Terminal head dipping	
	F	*p*	F	*p*	F	*p*	F	*p*
HA Vs LA	43.918	<0.001	13.118	<0.01	61.900	<0.0001	96.800	<0.0001

behavioural outcomes	Correlations (R coefficients)			
	Total head dipping	Proximal head dipping	Distal head dipping	Terminal head dipping
% Time in OA	0.7722	0.5359	0.7895	0.8460

Table 4.2: Statistical analysis and comparisons of the different parameters measured in the EPM. *LA rats characterised based on the percentage of time spent in the OA over the 5 min sessions displayed higher occurrence of head dipping in all the territories of the OA as compared to HA rats. The time spent in OA was positively correlated with the head dippings in all the territories of the open arms (OA).*

These data suggest that our variable of choice to measure trait anxiety, namely the percentage of time spent in the open arms, represents the constellation of anxiety-related behaviours measured on the EPM, therefore no further analyses involving head dipping were conducted.

In experiment 2, sign tracker (ST) rats, trained for five sessions in the autoshaping task progressively developed more Pavlovian approaches (contacts) to the lever / light compound CS than goal trackers (GT) *[main effect of group: $F(1,10) = 70.619$, $p < 0.0001$; of session: $F(4,40) = 19.255$, $p < 0.0001$; and group x session interaction: $F(4,40) = 19.97$, $p < 0.0001$]* (**Fig. 4.3 A**). In contrast GT allocated responding to the goal in that they made progressively higher numbers of head entries in the food magazine, as compared to ST rats *[main effect of group: $F(1,10) = 70.296$, $p < 0.0001$; of session: $F(4,40) = 3.8833$, $p < 0.01$; and group x session interaction: $F(4,40) = 8.0570$, $p < 0.0001$]* (**Fig. 4.3 A**).

In the rat Gambling task, good decision makers (GDM) rats displayed a higher percentage of advantageous choices over test session than bad decision makers (BDM) rats *[main effect of group: $F(1,10) = 241.05$, $p < 0.0001$]* (**Fig. 4.3 B**). Finally, HP rats preferred saccharin to water much more than LP rats did *[main effect of group: $F(1,10) = 94.524$, $p < 0.0001$]* (**Fig. 4.3 C**).

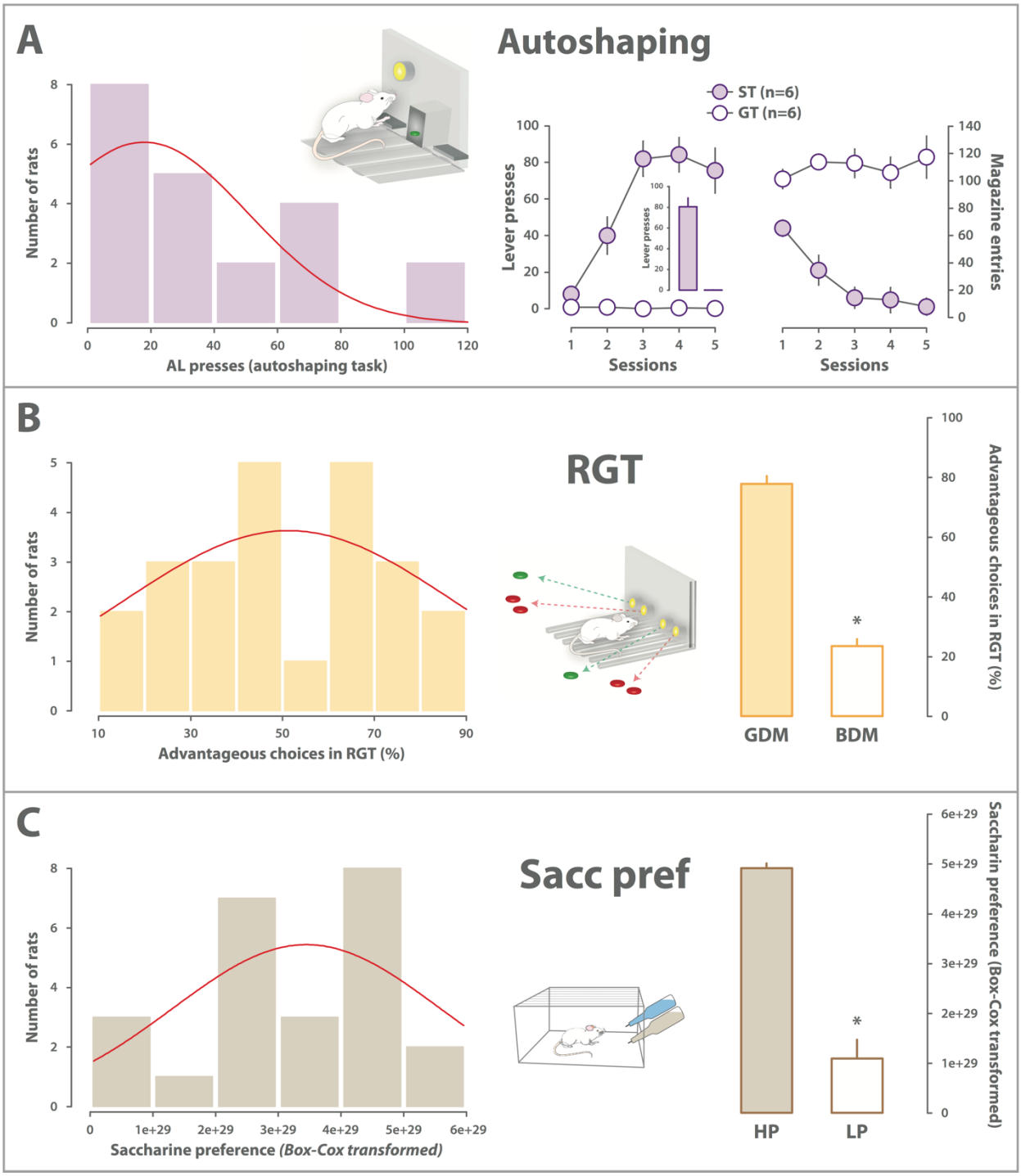


Figure 4.3: Characterisation of the behavioural traits assessed in experiment 2: Sign tracking / decision-making / sensitivity to natural reward. A) *Sign tracker (ST) rats displayed a higher level of responding on the cue-associated lever and lower magazine entries as compared to goal trackers (GT) GT.* ***B)*** *Good decision makers (GDM) displayed a higher percentage of advantageous choices in the RGT test session as compared to poor decision makers (PDM).* ***C)*** *High saccharin preferring rats (HP) drank a higher percentage of saccharin over water as compared to LP rats. The distribution of the population for each traits with regards to the segregative parameter followed a normal distribution at the exception of saccharine preference which had to be subjected to a Box-Cox transformation (red curves show the expected normal distributions).[*: p<0.05]*

The different behavioural dimensions investigated here were double dissociable in that dimensional analyses performed on the behavioural scores of the different tests carried-out in experiments 1 and 2 independently, did not correlate with one another (**Fig. 4.4**), thereby confirming that they represent distinct psychological behavioural traits and underlying constructs.

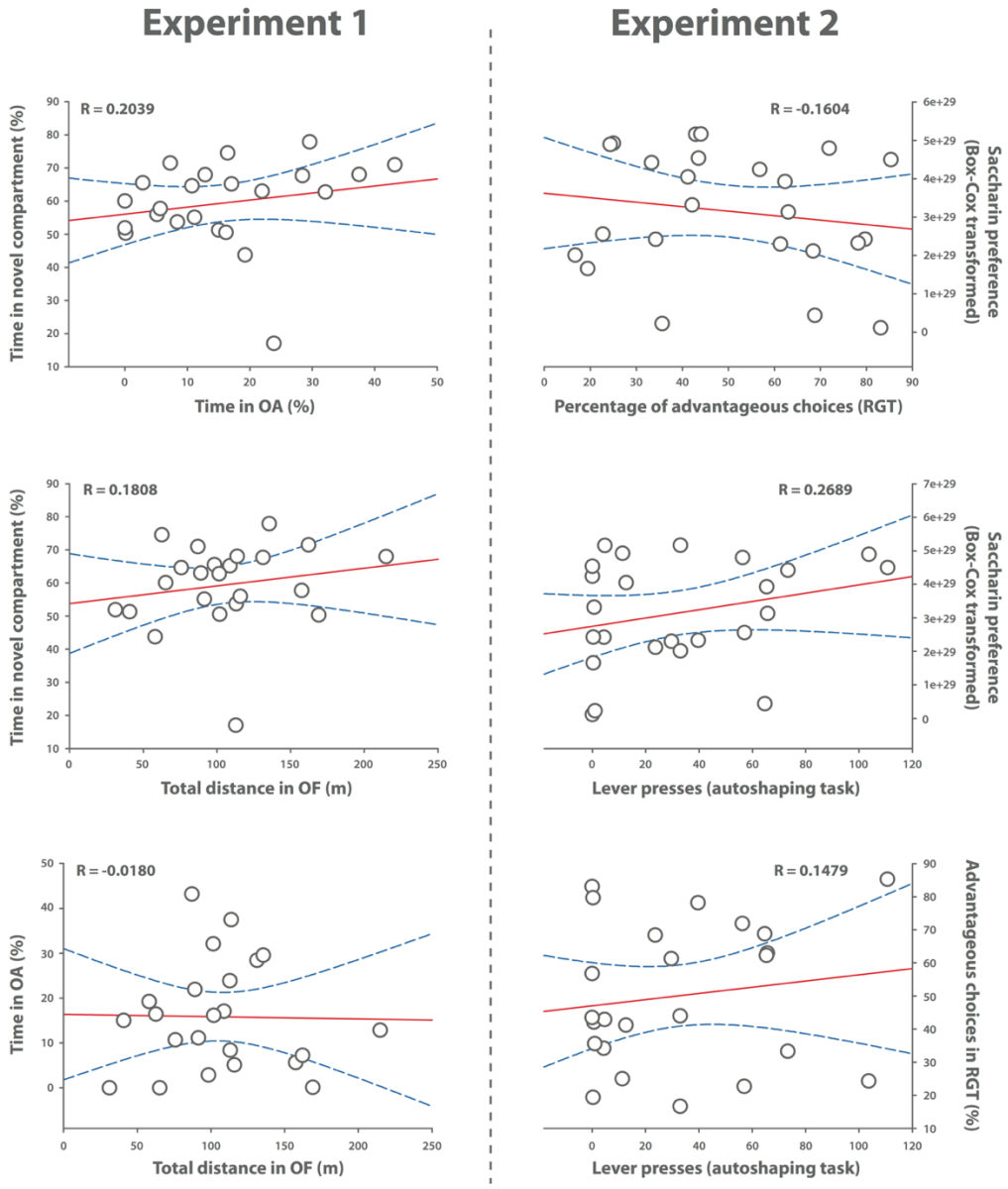


Figure 4.4: The different behavioural traits under investigation did not correlate. *Correlations between behavioural dimensions assessed in experiment 1 (left panel) and experiment 2 (right panel).*

First, we investigated the contribution of each trait on the emergence of incentive habits and subsequently the vulnerability to develop compulsive heroin seeking behaviour.

2. Contribution of the behavioural traits to the emergence of incentive habits

Following the characterisation of the behavioural traits previously described, rats were trained to self-administer heroin under the same schedule as described in Chapter 3 (**Fig. 4.5**).

We analysed each phase of the overall schedule independently. This enabled a more thorough investigation into the potential contribution of each behavioural trait to the propensity to self-administer heroin, to acquire high levels of heroin seeking, to the individual sensitivity to conditioned reinforcement and the associated vulnerability to develop incentive habits for heroin (**Fig. 4.5** and **Table 4.3** for statistical analyses).

The analysis of the acquisition of heroin self-administration under continuous reinforcement (**Fig. 4.5** and AL presses FR-1 sessions in **Table 4.3**) revealed that none of the behavioural traits tested predicted a differential propensity to acquire heroin intake at the dose used here.

Similarly, the analysis of the increase in lever pressing in response to the increasing duration of the intervals under fixed interval schedules of reinforcement confirmed that, apart from anxiety trait, which will be further discussed below, none of the traits predicted a differential propensity to acquire high rates of drug heroin seeking behaviour (**Fig. 4.5** and **Table 4.3**).

HA rats displayed a higher increase in drug seeking in response to the increase in the duration of the intervals under FI schedules of reinforcement *[main effect of group: $F(1,10) = 1.525$, $p > 0.05$, session; $F(7,70) = 31.634$, $p<0.0001$ and group x session interaction: $F(7,70) = 2.246$, $p<0.05$]* (**Fig. 4.5** and **Table 4.3**).

However, high anxiety trait did not predict an increased sensitivity to the conditioned reinforcing properties of the heroin-paired CS when introduced contingently upon responding under a SOR. Indeed, HA rats did not differ (at least statistically) from LA rats in their increase in heroin seeking relative to FI15 performance both overall and during the first interval during the first sessions of SOR (**Fig. 4.5, 4.6** and **Table 4.3 and 4.4**).

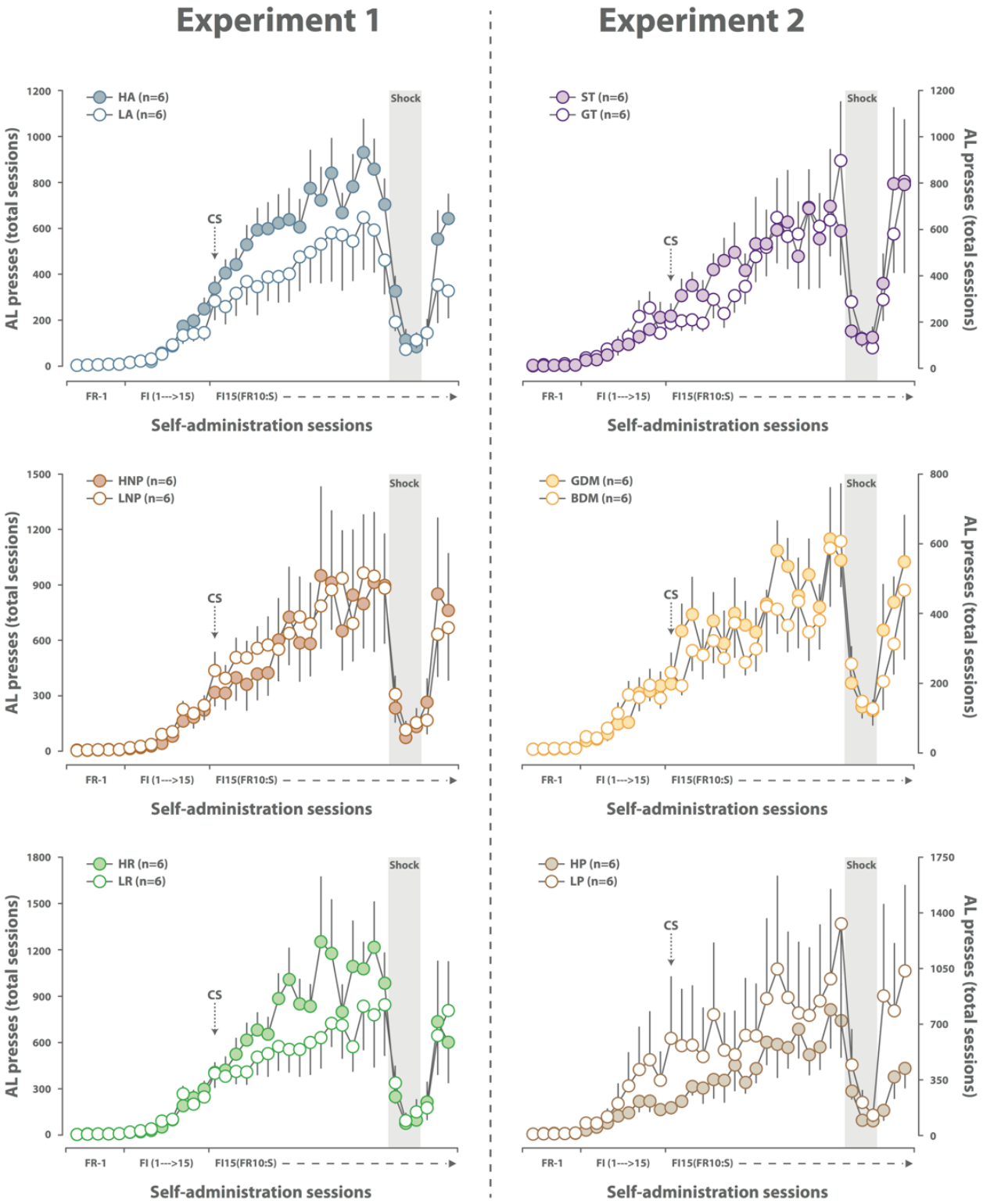


Figure 4.5: Analysis of the influence of potential behavioural markers of individual vulnerability to develop high levels of heroin seeking and compulsivity on the different stages of heroin seeking history. *None of the behavioural traits predicted a differential propensity to acquire heroin self-administration. HA rats were more prone to acquire heroin seeking under fixed interval schedules of reinforcement of increasing duration whereas sign trackers and HR rats were more prone than their counterparts to acquire cue-controlled heroin seeking and develop incentive habits, respectively.*

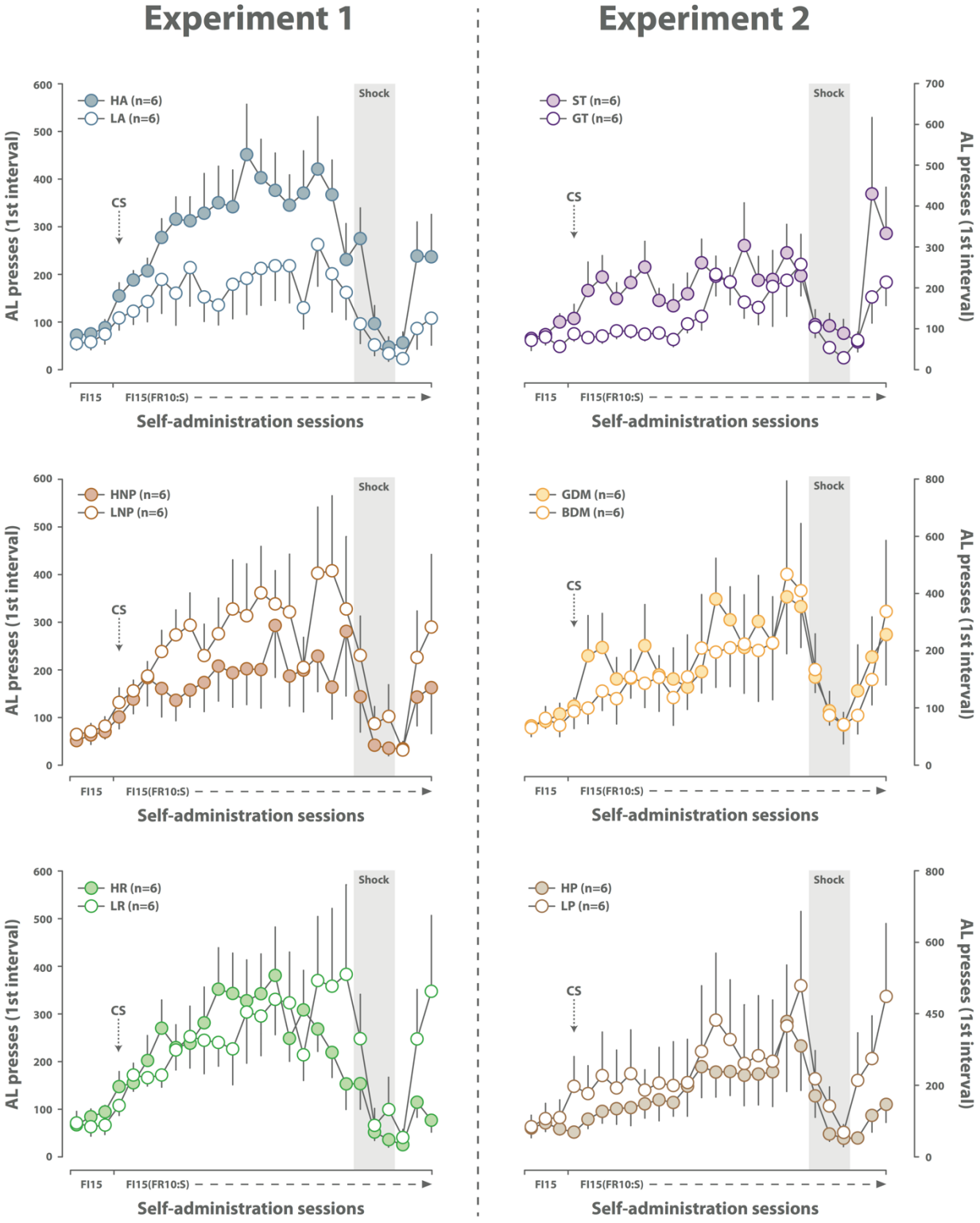


Figure 4.6: Analysis of the influence of potential behavioural markers of individual vulnerability to the sensitivity to conditioned reinforcement and the vulnerability to develop compulsive heroin seeking incentive habits, as measured during the first interval of SOR sessions with or without contingent punishment.

This is in contrast with the apparent effect of sign tracking, decision making and locomotor reactivity to novelty which all tend to predict a differential sensitivity to the conditioned reinforcing properties of the heroin-paired CS early on or a differential development of incentive habits, both during the entire session and the first interval of SOR sessions as compared with FI15 sessions (**Fig. 4.5, 4.6**). While no between-group effects were observed in an overall statistical design (**see Table 4.3 and 4.4**), the lack of power of the overall mixed design did not enable clear conclusions to be made with regards to specific behavioural traits, especially for the cases in which the F value suggested a clear difference between the means.

In order to circumvent this lack of power, the specific increase in responding upon introduction of the SOR (first three sessions) was compared, for each specific phenotype, to the level of responding under FI15 for both the overall session (**Fig. 4.5**) and the first interval (**Fig. 4.6**). The detailed statistical results are presented in **Table 4.4**.

Thus, HA and LA rats both showed sensitivity to the conditioned reinforcing properties of the CS as revealed by an immediate increase in responding under SOR as compared to FI15, a robust increase in responding that both groups maintained across sessions. Decision making and locomotor reactivity to novelty predicted a differential sensitivity to the early conditioned reinforcing properties of the CS. There was a marked difference between ST and GT in their sensitivity to the introduction of conditioned reinforcement, whereby increased responding under SOR as compared to FI-15 was only displayed by ST. however, as mentioned, the lack of statistical power lead to a non-significant effect (**Table 4.4**).

	1st block of SOR Vs FI-15			
	1st Int AL		Total AL	
	F	*p*	F	*p*
HA	86.35	<0.0012	18.881	<0.01
LA	15.28	<0.05	7.27	<0.05
HNP	16.084	<0.05	9.475	<0.05
LNP	93.024	<0.0012	16.451	<0.01
HR	50.827	<0.01	19.961	<0.01
LR	37.068	ns	11.372	<0.05
GT	<1	ns	<1	ns
ST	5.597	0.064	10.83	<0.05
GDM	6.421	ns	6.084	ns
BDM	6.715	<0.05	43.527	<0.01
HP	<1	ns	<1	ns
LP	3.483	ns	1.767	ns

Table 4.4: Influence of behavioural traits of putative vulnerability to the sensitivity to the conditioned reinforcing properties of the CS. *Statistical analyses of the responses in the first block of 3 sessions of SOR as compared to the 3 sessions of FI-15 schedule of reinforcment. Sign tracking, decision making and locomotor reactivity to novelty predicted differential sensitivity to the conditioned properties of the CS. [ns: non-significant; significance was set at p < 0.05].*

Categorical factors	Effects	AL presses FR-1 sessions		AL presses FI-1 to FI-15 sessions		Sensitivity to CR AL (total) (FI-15 block Vs 1st SOR block)		Sensitivity to CR AL (1st interval) (FI-15 block Vs 1st SOR block)		AL (total) SOR		AL (1st interval) SOR		Dvpt of incentive habit AL (total) (SOR blocks Vs FI-15 block)		Dvpt of incentive habit AL (1st interval) (SOR blocks Vs FI-15 block)	
		F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
HA Vs LA	groups	<1	ns	1.525	ns	1.577	ns	1.859	ns	1.515	ns	3.654	ns	1.54	ns	3.789	ns
	sessions/blocks	16.653	<0.0001	31.634	<0.0001	23.261	<0.001	73.407	<0.0001	6.951	<0.001	3.443	<0.01	14.45	<0.001	11.198	<0.0001
	interaction	<1	ns	2.246	<0.05	<1	ns	4.813	ns	<1	ns	1.055	ns	<1	ns	<1	ns
HNP Vs LNP	groups	2.624	ns	1.005	ns	<1	ns	<1	ns	<1	ns	1.129	ns	<1	ns	1.201	ns
	sessions/blocks	7.916	<0.0001	25.18	<0.0001	25.686	<0.001	58.037	<0.0001	4.581	<0.01	2.031	<0.01	7.921	<0.0001	6.975	<0.0001
	interaction	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns
HR Vs LR	groups	<1	ns	<1	ns	<1	ns	<1	ns	1.442	ns	<1	ns	1.678	ns	<1	ns
	sessions/blocks	16.507	<0.0001	45.299	<0.0001	30.635	<0.001	86.499	<0.0001	4.668	<0.001	2.192	<0.001	9.492	<0.0001	9.591	<0.0001
	interaction	<1	ns	1.766	ns	<1	ns	<1	ns	<1	ns	1.346	ns	1.103	ns	<1	ns
GT Vs ST	groups	1.611	ns	<1	ns	<1	ns	4.276	ns	<1	ns	2.13	ns	<1	ns	2.704	ns
	sessions/blocks	<1	ns	14.499	<0.0001	2.708	ns	5.632	<0.05	6.049	<0.001	4.87	<0.001	10.05	<0.0001	9.64	<0.0001
	interaction	1.402	ns	2.081	ns	3.643	ns	3.037	ns	<1	ns	1.22	ns	<1	ns	1.172	ns
GDM Vs BDM	groups	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns
	sessions/blocks	2.322	ns	18.954	<0.0001	13.421	<0.01	10.943	<0.01	4.883	<0.001	5.329	<0.001	9.81	<0.0001	6.702	<0.0001
	interaction	<1	ns	1.469	ns	1.396	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns
HP Vs LP	groups	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns
	sessions/blocks	7.134	<0.01	4.018	<0.001	2.053	ns	4.008	ns	6.074	<0.001	4.203	<0.001	7.683	<0.0001	3.93	<0.0001
	interaction	<1	ns	<1	ns	<1	ns	2.028	ns	<1	ns	<1	ns	<1	ns	<1	ns

Table 4.3: Main effects of ANOVAs carried-out with each putative behavioural trait of vulnerability to heroin addiction as between subject factor and various phases of heroin self-administation history as within-subject factor. *No main effect of groups was observed for the acquisition of heroin self-administation under continous reinforcement. However, HA rats displayed a higher propensity to seek cocaine under FI schedules of increasing duration than LA rats. Finally, several trends towards a differential response to conditioned reinforcement were observed between ST and GT rats in addition to GDM and PDM and HP and LP rats that require further analysis due to the relatively low power of the present analyses. [ns: non-significant; significance was set up to p<0.05].*

The lack of sensitivity to the conditioned reinforcing properties of the CS displayed by HP and LP rats might account for a marked inter-individual difference in responding, putatively triggered by outlier individuals.

Finally, ST rats displayed a trend towards being more resistant to punishment than GT rats over the last two drug free intervals of the three punished sessions (**Fig. 4.6**) [main effect of group: $F(1,10) = 2.977$, $p = 0.1$, session: $F(1,10) = 4.219$, $p > 0.05$; and group x session interaction: $F(1,10) < 1$].

Importantly, the differences and trends observed here were specific to the instrumental responses since, no differences between groups were observed for other key behavioural variables including the total number of heroin infusions or CSs received, and the total number of AL presses displayed throughout the entire experiment (**Table 4.5**, top panel). This lack of between-subject differences was further confirmed at the population level by a lack of correlation between each of the behavioural dimensions and the self-administration variables (**Table 4.5**, bottom panel).

behavioural outcomes	One-way ANOVAs											
	HA vs LA		HNP Vs LNP		HR Vs LR		GT Vs ST		GDM Vs BDM		HP Vs LP	
	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Total infusions	<1	ns	<1	ns	<1	ns	1.983	ns	1.641	ns	<1	ns
Total AL presses	1.832	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns
Total CSs	1.723	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns

behavioural outcomes	Correlations (R coefficients)					
	EPM score	NPP score	OF score	Autoshaping score	RGT score	Sacc preference score
Total infusions	-0.3241	-0.1513	0.1501	0.3793	0.0025	0.2620
Total AL presses	-0.2349	-0.0678	0.2520	-0.1056	0.1591	-0.1730
Total CSs	-0.2302	-0.0677	0.2659	-0.0955	0.1668	-0.0095

Table 4.5: Between-subject and dimensional analyses aiming to further characterise the potential predictive properties of putative behavioural traits of vulnerability on heroin self-adminsitation and heroin seeking-related variables. *No difference between groups was observed in the total heroin infusions or CSs received, or in the total instrumental behaviour deployed (AL presses) throughout the entire self-administration history [ns: non-significant].*

Overall, these data suggest that the behavioural traits investigated in this study do not predict a differential development of incentive habits for heroin.

3. Analysis of the behavioural markers of vulnerability to compulsive heroin seeking

The contribution of behavioural traits to individual vulnerability to develop compulsive heroin seeking behaviour was further investigated, focusing specifically on the peri-punishment sessions, including the three pre-shock baseline sessions, the three shock sessions and the three post-shock sessions.

Levels of responding (entire sessions and first intervals) over the three shock sessions and the three post-shock session as well as the number of shocks received in the first interval were independently analysed with groups as categorical factors. No differences were observed between groups in the behavioural outcomes over the punished sessions and during the post-punishment baseline except for HA rats which responded more than LA rats in the first punished session (entire sessions or first intervals) (**Table 4.6**).

Since incentive habits have been previously shown (see chapter 3) to result in an aberrant engagement in drug seeking in anticipation of punishment during the first half of the interval of punished sessions and an increased propensity to resume pre-punishment seeking levels after cessation of contingent deliver of footshocks, the potential contribution of behavioural traits of vulnerability to a differential propensity to display these behavioural features was further investigated.

Categorical factors	Effects	**Anticipation**: % AL 8 and 7 min (1st int) shock Vs baseline		**Recovery**: last 2 skock Vs last 2 post-shock	
		F	***p***	**F**	***p***
HA Vs LA	groups	2.461	ns	2.188	ns
	blocks	9.158	<0.05	8.472	<0.05
	interaction	3.765	ns	2.18	ns
HNP Vs LNP	groups	<1	ns	<1	ns
	sessions	8.197	<0.05	6.116	<0.05
	interaction	1.338	ns	<1	ns
HR Vs LR	groups	<1	ns	1.84	ns
	blocks	8.719	<0.05	7.111	<0.05
	interaction	<1	ns	2.662	ns
GT Vs ST	groups	<1	ns	2.74	ns
	blocks	5.683	<0.05	6.056	<0.05
	interaction	<1	ns	<1	ns
GDM Vs BDM	groups	<1	ns	<1	ns
	blocks	8.811	<0.05	5.452	<0.05
	interaction	<1	ns	<1	ns
HP Vs LP	groups	<1	ns	1.938	ns
	blocks	5.645	<0.05	6.551	<0.05
	interaction	<1	ns	2.027	ns

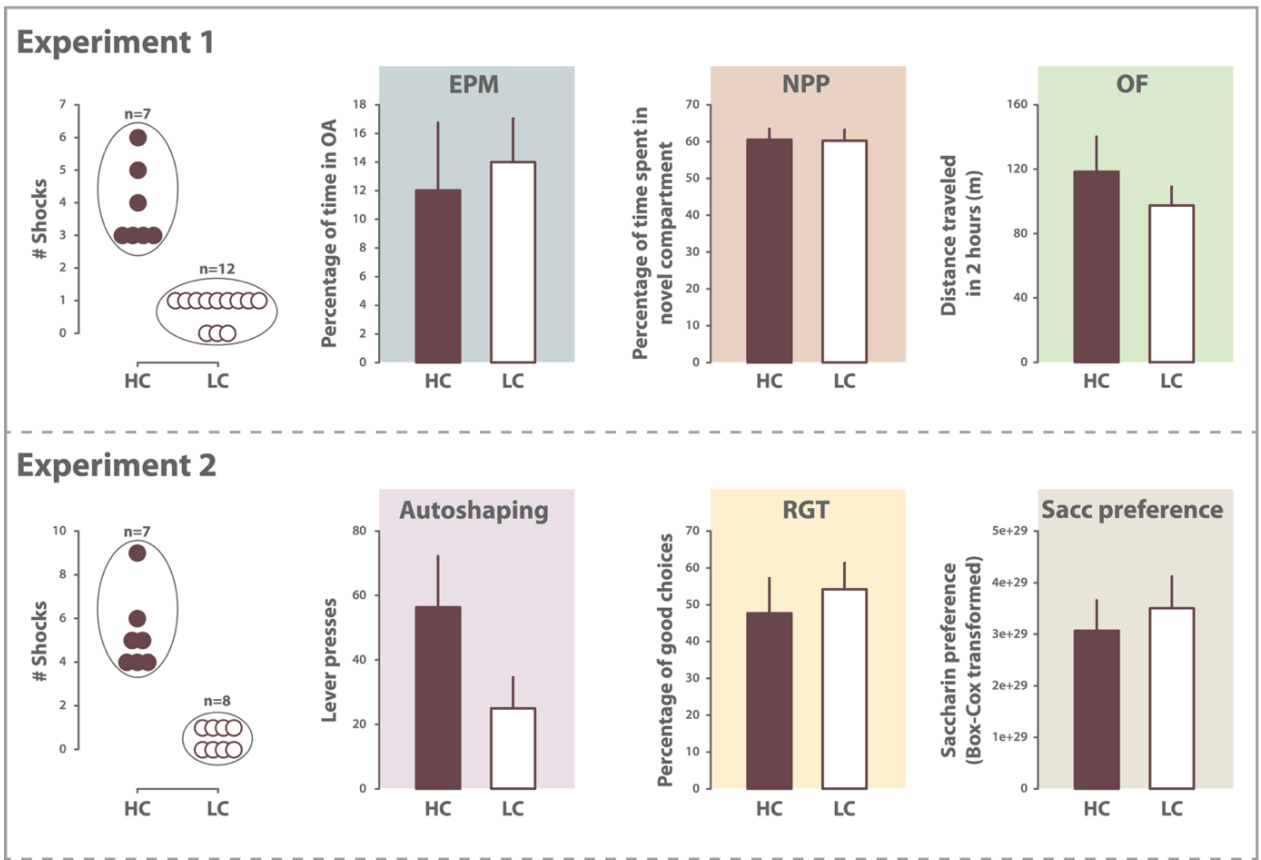
Table 4.7: Influence of behavioural traits that putatively confer vulnerability to the aberrant increase in responding in anticipation of punishment and the recovery of heroin seeking after its cessation. *Statistical analyses of the percentage of responses in the first 8 min and the last 7 min of the first intervals of the punished sessions as compared to baseline and the level of responding in the first interval displayed after punishment revealed that none of the behavioural traits under investigation predicted an increased propensity to display aberrant drug seeking behaviour prior to, or following punishment. [ns: non-significant; significance was set at $p < 0.05$].*

Categorical factors	Effects	Baseline sessions - total AL presses		Baseline sessions - AL presses 1st interval		Shock sessions - total AL presses		Shock sessions - AL presses 1st interval		Post shock sessions - total AL presses		Post shock sessions - AL presses 1st interval		# Shocks	
		F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	p
HA Vs LA	groups	1.526	ns	1.519	ns	<1	ns	2.87	ns	1.66	ns	2.436	ns	<1	ns
	sessions	4.579	<0.05	6.29	<0.01	30.24	<0.001	24.1	<0.001	14.43	<0.001	8.576	<0.01		
	interaction	<1	ns	<1	ns	5.64	<0.05	8.08	<0.01	2.57	ns	1.562	ns		
HNP Vs LNP	groups	<1	ns	<1	ns	<1	ns	1.062	ns	<1	ns	<1	ns	<1	ns
	sessions	<1	ns	<1	ns	8.986	<0.01	6.316	<0.01	8.808	<0.01	5.844	<0.01		
	interaction	<1	ns	3.107	ns	<1	ns	<1	ns	<1	ns	<1	ns		
HR Vs LR	groups	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	2.215	ns	<1	ns
	sessions	<1	ns	<1	ns	16.82	<0.001	8.684	<0.01	8.897	<0.01	6.261	<0.01		
	interaction	1.081	ns	<1	ns	<1	ns	<1	ns	<1	ns	2.787	ns		
GT Vs ST	groups	<1	ns	<1	ns	<1	ns	1.35	ns	<1	ns	1.308	ns	4.812	ns
	sessions	2.675	ns	<1	ns	9.155	<0.01	5.424	<0.05	7.745	<0.01	5.988	<0.01		
	interaction	3.666	<0.05	<1	ns	4.861	<0.05	2.007	ns	<1	ns	1.521	ns		
GDM Vs BDM	groups	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns
	sessions	3.421	ns	2.248	ns	10.31	<0.01	6.788	<0.01	4.367	<0.05	4.176	<0.05		
	interaction	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns	<1	ns		
HP Vs LP	groups	<1	ns	<1	ns	<1	ns	<1	ns	1.269	ns	1.908	ns	1.952	ns
	sessions	6.124	<0.01	7.111	<0.01	6.811	<0.01	10.591	<0.01	2.691	ns	5.288	<0.05		
	interaction	2.698	ns	2.758	ns	<1	ns	<1	ns	1.377	ns	1.281	ns		

Table 4.6: Results of the ANOVAs performed for each behavioural trait on the punishment-related variables pertaining to the characterisation of compulsive heroin seeking. *While no main effect of group was observed, the group x session interaction revealed within the punished sessions for high anxiety (HA) vs low anxiety (LA) rats confirm that HA rats indeed resisted much more to punishment during the first session than LA rats, as represetend in Fig. 4.5. Similarly, the group x time interaction obserfved during the shock sessions reveals that ST rats resusted to punishmen more than GT rats during the first interval of the session, thereby suggesting that compulsive heroin seeking behaviour may be associated with sign tracking [ns: non-significant; significance was set at $p < 0.05$].*

As presented in **Table 4.7**, no difference was observed between groups in the maladaptive responses displayed prior to punishment or during the last two post-punishment sessions (as compared to the last two punished sessions, as described in Chapter 3). However, a main block effect was significant for all the groups revealing that irrespective of trait rats resumed higher levels of heroin seeking following cessation of punishment, as shown in Chapter 3.

To better to characterise the behavioural profile of compulsive and non-compulsive rats, the population of each experiment was subsequently segregated into three non-overlapping groups (namely HC, IC and LC) based on the number of foot-shocks received over the last two punished sessions (**Fig 4.7**). Thus, high compulsive (HC) rats did not differ from low compulsive (LC) rats in



	One-way ANOVAs											
	EPM score		NPP score		OF score		Autoshaping score		RGT score		Sacc pref score	
	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
HC Vs LC	<1	ns	<1	ns	<1	ns	3.048	ns	<1	ns	<1	ns

Figure 4.7: retrospective behavioural characterisation of rats identified as high and low compulsive for heroin seeking. *HC and LC rats did not differ in their behavioural performance in any investigated task. [ns: non-significant; significance was set at $p < 0.05$]. Still HC rats tended to display higher sign-tracking levels than LC rats in the autoshaping procedure.*

the putative behavioural markers of vulnerability prior to heroin exposure, at the exception of a trend in sign tracking (HC rats displayed much higher levels, albeit non statistically significant, than LC rats) (**Fig 4.7**)

Altogether, these data suggested that the behavioural traits investigated in this study do not predict the development of behavioural features of the multifaceted nature of compulsive heroin seeking habits.

Discussion

This study investigated the potential predictive value of several behavioural traits to the individual vulnerability to develop incentive habits for heroin and associated compulsivity.

Two cohorts of rats were enrolled in complementary longitudinal studies wherein individuals were tested on several tasks enabling the characterisation of individual differences in sign tracking, anxiety, decision making, sensation seeking, novelty seeking and reward sensitivity in a drug naïve state. Subsequently animals' propensity to develop incentive habits for heroin and compulsive heroin seeking were measured.

The marked inter-individual differences observed in the tasks used here to identify the selected behavioural traits did not correlate. This is in line with previous data [174, 192, 210, 212, 314] and supports the construct validity of the present approach which aimed to characterise several non-overlapping behavioural features in the rats that have been suggested to reflect distinct personality traits in humans [267-269].

None of the behavioural dimensions and associated traits predicted the propensity to acquire heroin self-administration under continuous reinforcement. Additionally, the present study did not identify behavioural markers of vulnerability to compulsive heroin seeking behaviour robust enough to survive the statistical threshold of the mixed design used here. These data contrast with the wealth of literature on the behavioural markers of vulnerability to acquire cocaine use, escalate cocaine intake, develop high motivation for cocaine or switch to compulsive cocaine intake. Thus, it seems that factors that confer vulnerability to acquire heroin self-administration and switch to compulsive heroin seeking behaviour differ, at least partially, from those identified for cocaine, as discussed in the introduction of this chapter.

This apparent discrepancy is likely due to the lack of power in the present study, as strong trends were observed that warrant further investigations. Previous studies always measured instrumental responding with drug on board, and there is currently no available data on the vulnerability to compulsively seek (not self-administer) drugs. Thus, the characterisation of compulsive cocaine seeking habits in chapter 3 will also offer new avenues to investigate whether the behavioural factors that predict different aspects of drug-related behaviours also predict the vulnerability to compulsive cocaine seeking.

Since the potential contribution of high impulsivity trait to the vulnerability to develop compulsive heroin seeking behaviour was not measured here, the role of impulse control deficits remains an open question. Indeed, while high impulsivity trait, as measured by high levels of premature responses in the 5-choices serial reaction time task, predicts both the vulnerability to develop compulsive cocaine self-administration [192] and escalation of cocaine intake [297] it does not contribute to an increased vulnerability to escalate heroin intake [189].

Nevertheless, the present data demonstrate that high anxiety trait predicted an increased propensity to engage in drug seeking under Fixed interval schedules of reinforcement. This observation suggests that high anxious rats were actually more motivated than LA rats to respond for heroin, as fixed ratio schedules of reinforcement measure only the reinforcing properties of the drug [165]. HA rats were also more resistant to the punishment of ongoing heroin seeking behaviour during the punished sessions. Actually, HA rats showed an increased resistance to punishment during the first shock session, but they eventually decreased their seeking response to the level displayed by LA rats by the third session. This apparent increased resistance to punishment may be attributable to a higher level of responding under SOR prior to punishment displayed by HA rats as compared to LA rats. Therefore, this observation will need to be replicated, alongside the apparent increased propensity HA rats had to develop high levels of responding under conditioned reinforcement for heroin, in order fully to conclude that high anxiety trait indeed predicts an increased vulnerability to compulsive heroin seeking.

Nevertheless the observation that HA rats are more motivated for heroin in comparison to LA rats under FI schedules of reinforcement, and may be more vulnerable to compulsive heroin seeking behaviour is in marked contrast with the evidence that HA rats are more vulnerable to

escalate cocaine, but not heroin intake during extended access [209]. This further exemplifies the notion that escalation of drug intake does not reflect the compulsive nature of drug addiction [193, 315, 316], as captured by inter-individual differences in resistance to punishment of the seeking responses, such as those measured here. It also offers support to the self-medication hypothesis of opiates addiction whereby the psychoaffective state in which one individual is when they initiate drug use, aiming to medicate an internal distress, such as high anxiety, may represent a gateway for the subsequent development of compulsive drug seeking habits [107, 108, 317].

Interestingly, it remains unknown why high anxiety trait predicts an increased propensity to escalate cocaine intake. Cocaine has anxiolytic properties when administered acutely and at low doses [318] but anxiogenic properties when administered at high doses [319]. Therefore, HA rats may well initiate cocaine self-administration as a self-medication of high anxiety and their subsequent escalation could reflect either an enhancement of the anxiolytic properties, or a tolerance to the anxiogenic properties of cocaine. Clearly such mechanisms very likely play little, if any, role in the apparent high levels of responding for heroin under SOR as observed in the present study. Further research is warranted better to understand how anxiety, stress and opiate-induced opponent processes interact in promoting high levels of incentive habits for heroin and compulsive heroin seeking behaviour.

The other behavioural trait that was shown to differentially predict an increased vulnerability to develop compulsive heroin seeking habits was sign tracking. Sign trackers were shown to be more sensitive to the conditioned reinforcing properties of the heroin-paired CS than goal trackers, who actually displayed no increase in responding upon introduction of the SOR. The increased sensitivity to conditioned reinforcement by a drug-paired cue observed in ST rats was expected since they attribute higher incentive value to CSs [320]. However, this is the first demonstration of an increased sensitivity to the conditioned reinforcing properties of a heroin-paired cue in ST rats engaged in heroin seeking over prolonged periods of time. Although, this trend was only observed at the onset of the conditioned reinforcement, i.e. the first three SOR sessions and not over the subsequent ones. This observation suggests that goal-trackers eventually learnt to respond for the conditioned reinforcing properties of the heroin-paired cue

such that, at the dimensional level, high incentive salience for a reward-associated cue as displayed by ST rats in the autoshaping task did not predict higher levels of responding in heroin seeking under Fixed interval schedules of reinforcement, and especially under SOR. The attribution of incentive values to the cue in the autoshaping task might be directly related to the motivational and sensory properties of the reward, in this case, food pellet. In the phase of acquisition of heroin self-administration (from FR-1 to FI-15), other CSs are presented contingently with each heroin infusion and acquire a different incentive value to the one associated with the food pellet. Thus, the attribution of incentive salience to stimuli in the same individuals could be different depending on the nature of the reinforcer, i.e. how individuals process the motivational value of the reinforcer. In order to further test that hypothesis, it would be interesting to investigate whether ST trackers, as compared to GT, would display higher rate of responding when subjected to SOR schedule of reinforcement for the same reinforcer as the one used in the autoshaping task (i.e. food pellet).

Interestingly, if ST rats did display a higher propensity to respond under a SOR for heroin than GT rats, their overall level of responding was relatively low as compared to the one displayed by HA rats. This also suggested that other factors than the individual propensity to ascribe incentive motivational value to CSs may contribute to the response enhancing effects of this cue when used as a conditioned reinforcer.

ST rats seem to be slightly more resistant to punishment than GT rats, based on the higher level of responding during the first drug-free interval of the last two punishment sessions. This difference did not come out as statistically significant, but again, this may be attributable to the relatively low power of the overall design. Nevertheless, this is in agreement with the symmetrical evidence that high compulsive rats tend to have a much higher level of sign tracking prior to drug exposure than non-compulsive rats. This may represent the fact that the increased motivational value of the CS eventually contributes to a more rigid incentive habit, leading to DLS control over behaviour that cannot be completely disengaged in the face of adverse consequences, resulting in persistence in responding that is defined as compulsive in contemporary behavioural neuroscience. This requires further investigation, but such a conclusion would be in agreement with evidence that high alcohol preferring rats that develop

compulsive alcohol seeking behaviour specifically display an inability to disengage DLS control over responding in the face of punishment, as demonstrated recently by Dr. Chiara Giuliano in Dr. Belin's laboratory.

As observed for sign trackers, HR rats, characterised on their locomotor reactivity to novelty, displayed an increased sensitivity to the conditioned reinforcing properties of the heroin-paired CS, particularly when the drug was 'on-board' as differences were more pronounced across the entire session rather than the first interval. One potential interpretation of this observation is that HR rats are more sensitive to the novelty of the CS presented contingently upon responding and engage in instrumental novelty seeking. This could account for the differential performance during the first interval as HR rats in that drug-free interval progressively stop responding for the CS and fall below the level of responding displayed by LR rats. However, it could not account for the sustained higher level of responding across the several weeks of training observed for the entire session. One alternative interpretation is that the HR phenotype actually predicts an increased propensity to engage in lever pressing, and consequently acquire instrumental responding [321].

Inter-individual differences in the sensitivity to natural reward have been associated with various drug-related behaviours in a drug-dependent manner, e.g., differentially between psychostimulants and opiates. Although much of the published studies suggest a positive correlation between individual sensitivity to natural reward and an increased propensity to self-administer psychostimulants and alcohol [214, 311, 312, 322, but see 323], there is no such unequivocal relationship for heroin. Thus, using rat lines that were selectively bred for high (HiS) or low (LoS) saccharin intake [324], Carroll and colleagues showed that females, and to a lesser extent males, HiS demonstrated a greater acquisition of cocaine self-administration than LoS rats but no such difference was observed for heroin self-administration [325]. Here, if it were not for one outlier in the LP group that artificially brought the average of that group higher than the one of the HP group, no differences whatsoever were observed between these two populations stratified on their preference for saccharine. This suggests that the self-medication hypothesis discussed above in the context of anxiety does not necessarily involved a differential level of sensitivity to natural rewards. The lack of interest in other sources of reinforcement displayed by

individuals suffering from heroin addiction may therefore represent an adaptation to the chronic exposure to the drug. It may be the same for decision making deficits [71, 326] since the present study did not identify any convincing evidence that bad decision makers are more vulnerable than good decision makers to develop compulsive heroin seeking habits.

Surprisingly, none of the behavioural traits investigated here predicted the development of the aberrant peri-punishment responses that were shown to characterise incentive habits in chapter 3, namely, increase in responding during the first half of the punished intervals in anticipation of the loss of the opportunity to freely respond during the subsequent punished period and a quick recovery of heroin seeking after cessation of punishment. This may suggest that these adaptations may occur in response to drug exposure in all individuals exposed long enough to foraging for heroin under the control of the conditioned reinforcing properties of the drug-paired CSs.

Still this study offers new insights in the behavioural endophenotypes of vulnerability to compulsive heroin seeking habits. Clearly, when added to the wealth of data in the literature on those of vulnerability to compulsive cocaine, the vulnerability to the development of heroin addiction-like behaviour highlights the complex importance of the nature of the reinforcer. This study was conducted on relatively small populations which enabled to identify subgroups of n=6, which offer only limited statistical power in the context of mixed designs. It would therefore, be interesting to replicate these experiments using larger cohorts in order to confirm the observations discussed above.

CHAPTER 5: CELLULAR CORRELATES OF INCENTIVE HABITS AND COMPULSIVE DRUG SEEKING

Introduction

1. A neural systems perspective of compulsive drug seeking habits.

The development of incentive habits for cocaine has been shown, at the neural systems level, to be underlined by a progressive functional transition from a AcbC-pDMS network to a AcbC-aDLS network [202] in the control over behaviour. Dopaminergic and glutamatergic mechanisms in the aDLS interact to maintain well established, but not early acquired, cue-controlled drug seeking behaviour [145]. Causal manipulations in rats foraging for cocaine under SOR have further revealed that the functional recruitment of aDLS-dopamine dependent control over behaviour when drug seeking becomes habitual is dependent on dopamine-dependent interactions between the AcbC and the aDLS [146], potentially recruited by BLA-dependent glutamatergic mechanisms in the AcbC, but eventually maintained over prolonged periods of time by the CeA [245] (**Fig. 5.1**).

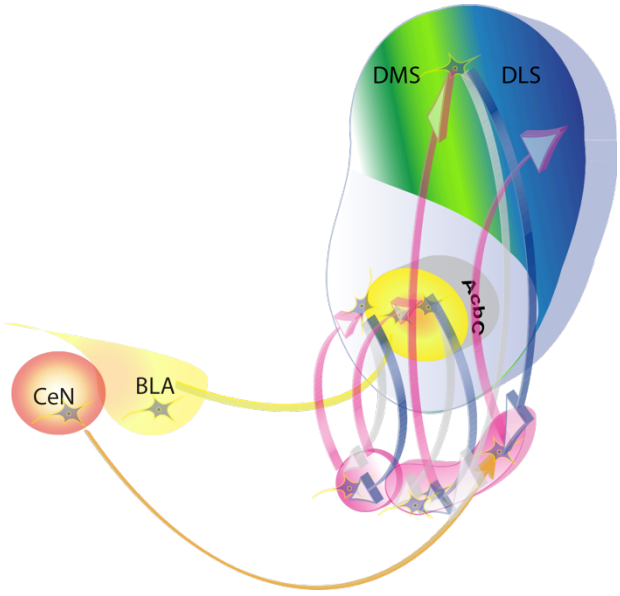


Figure 5.1: Neurobiological substrates surbserving the development of habitual cue-controlled cocaine seeking behaviour. *The acquisition of cocaine seeking under SOR, which is supposedly goal-directed, depends upon the interaction between the the BLA and the AcbC as well as dopamine-dependent mechanisms in the pDMS. However when cue-controlled cocaine seeking is instantiated as an incentive habit, it depends upon anterior DLS dopamine-dependent mechanisms, the recruitment of which depends on the AcbC and the striato-nigro-striatal (dopamine-dependent) ascending spiralling circuitry (involving the SNc). This functional recruitment of aDLS dopamine-dependent control over behaviour is triggered by the BLA but is eventually maintained by the CeA (CeN). [AcbC, Core of the Nucleus Accumbens ; BLA, Basolateral Amygdala; CeN central nucleus of amygdala ; VTA, Ventral Tegmental Area; SNc, Substantia Nigra pars compacta ; DLS, Dorsolateral Striatum, DMS: dorsomedial striatum (from 53)].*

The progressive functional recruitment of aDLS dopamine-dependent control over behaviour may be also be reflected by the neurobiological adaptations within the striatum to cocaine observed in non-human primates and rats over the history of drug self-administration.

Alterations in glucose metabolism [327], levels of DAT [328] and D2 receptors [329, 330] that are initially restricted to the ventral striatum progressively spread to the more lateral and dorsal parts of the striatum. Importantly, the alterations in DAT binding observed in the striatum over the course of drug exposure have hitherto been considered to reflect adaptations within dopaminergic neurons on the terminals of which DAT controls phasic dopamine transmission. However, DAT is also expressed on astrocytes in the striatum [28, 331], which it may be more involved in controlling tonic or volume dopamine transmission (**Fig. 5.2**).

It has been hypothesised that the recruitment of aDLS-dopamine dependent control over behaviour that mediates the development of drug seeking habits represents a key neurobiological mechanism towards the development of addiction [65, 142, 231]. Indeed, while it has been associated with craving in human individuals addicted to cocaine and compulsive cocaine seeking behaviour in rats [132, 332], dopamine release in the dorsal striatum has been shown to be triggered by presentation of drug-paired cues to recreational cocaine users who do not meet the DMS criteria for substance abuse or addiction [133]. These data, alongside the evidence that incentive habits develop in all individuals seeking cocaine or heroin under SOR (see chapter 3), therefore suggest that compulsivity in cocaine, and potentially heroin, addiction, may stem from a loss of control over these aDLS-dopamine dependent incentive habits. Such hypothesis is in agreement with the observations that human individuals addicted to drugs show a significantly enlarged DLS [333, 334] and a differential expression of D2 receptors [136] and dopamine transporter [335] within this structure as compared to drug naïve individuals.

Despite these recent insights into the neural systems associated with the development of cocaine seeking habits, little is known about the within (intrastriatal) and between- (cortico-striatal) systems cellular and molecular mechanisms by which these habits are maintained over prolonged periods of time, and sometimes despite negative consequences. In particular, the neural and cellular basis of compulsive heroin seeking habits have not been investigated yet.

However, importantly, the progressive recruitment of aDLS dopamine-dependent control over drug seeking, as measured as sensitivity of instrumental responding to intra aDLS infusions of the dopamine receptor antagonist α-flupenthixol after a prolonged history of training under SOR has also been shown to occur for heroin (unpublished data) [336]. Thus, similar intrastriatal

mechanisms may occur between cocaine and heroin during the development of incentive habits despite the clear different pharmacological targets of these drugs and the differential reliance of their reinforcing properties on the mesolimbic dopamine system [337-339].

This common dopaminergic mechanism in the establishment of aDLS-dependent cocaine and heroin seeking habits suggests that there may be some overlap in the neural and cellular factors that contribute to the development of compulsive seeking habits for cocaine and heroin, both within the striatum and the corticostriatal circuit (the function of which is dysregulated in addiction).

The top-down executive or inhibitory cortical control over striatal mechanisms has been shown to be impaired in drug addicts. Imaging studies in humans revealed that individuals who abuse drugs suffer from physical and functional alterations of areas of the prefrontal cortex [134, 340, 341] that are involved in inhibitory control, decision making and in the balance between goal-directed and habitual control over behaviour. Thus, the control over behaviour by A-O associations depends on pDMS and its interactions with the orbitofrontal cortex (OFC) and the prelimbic prefrontal cortex (PL) [238, 342, 343]. In contrast, control over instrumental responding by S-R associations depend on the aDLS and the infralimbic prefrontal cortex (IL) [238, 344, 345]. Dynamic interactions within these corticostriatal circuits, emphasising the role of the OFC and PL/IL functional balance have been shown in rodents to control the functional shift from DMS to DLS and associated transition from goal-directed behaviour to habits [346].

Interestingly, over the course of cocaine exposure in non-human primates, the progressive recruitment of the dorsolateral territories of the striatum is paralleled by a spread of neurobiological adaptations within the prefrontal cortex from the ventromedial prefrontal cortex to the orbital and the more dorsolateral cortices [347].

The entire corticostriatal circuitry may therefore contribute to the establishment of compulsive drug seeking habits. However, whether the intra-cortical transitions drive the intrastriatal ones or whether the former are an indirect consequence of the latter, which have been shown in rats, at least for cocaine, to be driven by intra-amygdala transitions [245], remains to be determined. Yet, deficits in synaptic plasticity and function within the prelimbic cortex have also been associated with compulsive cocaine intake [196] and cocaine seeking behaviour [198].

Chen and colleagues [198] showed that the PL hypoactivity observed in rats trained to self-administer cocaine over a protracted period of time was more pronounced in individuals displaying compulsive cocaine seeking behaviour. Optogenetic stimulation of the PL was shown to rescue its hypoactivity and prevent the expression of compulsive cocaine seeking [198].

Additionally, the insular cortex, which interacts with all the prefrontal, striatal and amygdalar regions involved in drug seeking habits or compulsive drug seeking, has also been shown in rats to contribute to high impulsivity trait [348] and the associated propensity to escalate cocaine intake [349] and develop compulsive behaviours [348]. In humans, the insula has been shown to be involved in decision making [350], but also craving [78, 351, 352] and impaired insight in individual addicted to drugs [76], suggesting it is an important neural locus of the vulnerability to compulsive drug seeking that requires to be investigated within a broad corticostriatal network.

Thus, in order to better characterise the neural correlates of compulsive heroin seeking habits, a hotspot analysis was carried using *in situ* hybridisation focusing on the immediate early gene ZIF-268, which is considered to be a marker of neuronal activity tightly linked to neuronal plasticity [353-355]. In order to delineate the neural and cellular correlates of the contribution of incentive habits to the emergence of compulsivity, the hotspot analysis was carried-out on brain sections obtained from rats exposed to punishment either after a history of SOR or FI15 schedule of reinforcement (as presented in chapter 3). This strategy not only helped to tease apart the differential functional recruitment of specific regions of the corticostriatal circuitry between incentive habits and habits but also offered a mapping of the “neural signature” of compulsive drug seeking behaviour.

In order to further probe the substrates of incentive habits and compulsivity, a candidate genes approach was selected to investigate the involvement of three underestimated endogenous regulatory systems converging onto the striatal synapse, namely the endocannabinoidergic, opioidergic and adenosinergic systems in the drug-induced neuroplasticity observed in the hotspot analysis.

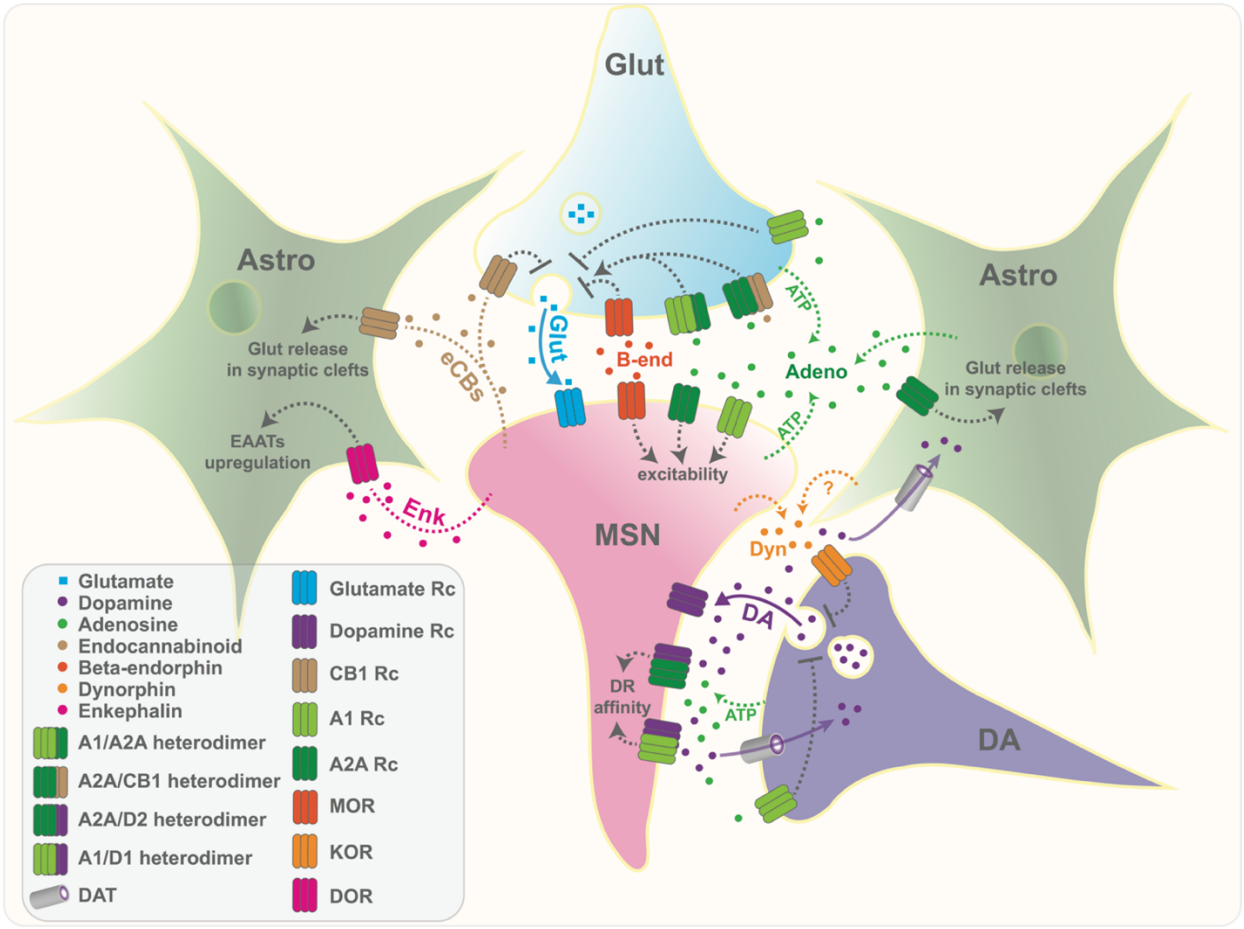


Figure 5.2: Between systems-mediated glutamate and dopamine signaling within the striatal tripartite synapse. *Dopamine is released by the terminal of dopaminergic neurons and bind to D1- and D2-like post-cynaptic receptors. DA is quickly taken up back into the presynaptic terminal by the dopamine transporter (DAT) which ensures the phasic nature of dopamine transmission. However, DAT is also expressed on striatal astrocytes on which he may control the spread of the volume transmission of DA. Adenosine is released by astrocytes or is transformed by extracellular enzymatic reactions from neuronal ATP within the synaptic cleft. A1 receptors are expressed presynaptically both on the dopaminergic and glutamatergic terminals where their activation inhibits neurotransmitter release. A1 and A2A receptors are expressed postsynaptically and modulate the neuronal excitability of MSNs. Adenosinergic receptors also have the ability to form heterodimers: on glutamatergic projections, activation of the heterodimers A1/A2A and A2A/CB1 modulates glutamate release (depending on the extracellular level of adenosine) and dimerization of postsynaptic A2A with D2, or A1 with D1, modulates negatively the affinity of dopaminergic receptors to dopamine. Activation of astrocytic A2A receptors enhances glutamate release from astrocytes. Postsynaptic endocannabinoids bind to CB1 receptors expressed both on presynaptic glutamatergic projections where they inhibit neurotransmitter release and on astrocytes where they increase glutamate release. Dynorphin, released by postsynaptic neurons (and potentially by astrocytes), binds to κ-opioid receptors expressed on dopaminergic terminals and their activation inhibits dopamine release. Post-synaptic Enkephalin binds to δ-opioid receptors which activation in astrocytes has been shown to up-regulate the expression of astrocytic glutamate transporters (EAATs). β-endorphin binds both pre and postsynaptically on μ-opioid receptors. Their activation on glutamatergic projection inhibits glutamate release while postsynaptically, it modulates negatively the neuronal excitability of MSNs. [astro: astrocyte; Glut: glutamate; DA: dopamine; ATP: adenosine triphosphate; Adeno: adenosine; eCBs: endocannabinoids; MSN: medium spiny neurons; DR: dopamine receptors; Dyn: dynorphin; D1: dopamine receptor D1; D2: dopamine receptor D2; Enk:enkephalin; B-end: beta-endorphin; A1: adenosine 1 receptor; A2A: adenosine 2a receptor; MOR: μ-opioid receptor; KOR: κ-opioid receptor; DOR: δ-opioid receptor; CB1: cannabinoid receptor 1; EAAT: excitatory amino acid transporter [from 235].*

2. Striatal molecular substrates of the functional recruitment of aDLS

a. Endocannabinoid system

Endogenous cannabinoids (eCB), synthetized from membrane lipids of cells, including post-synaptic neurons, act as retrograde neural messengers [356]. Anandamide and 2-arachidonoyl glycerol (2-AG) are the most studied eCB, they bind at cannabinoid-1 (CB1) and -2 (CB2) receptors, both Gi/0-protein-coupled. Within the striatum, CB1 receptors are strongly expressed in the ventral and the dorsal territories [357-359], where their activation inhibits the release of glutamate, GABA and acetylcholine [360], while it increases the firing rate of dopaminergic neurons and facilitates dopamine release in the nucleus accumbens through the inhibition of GABAergic interneurons [361-363] (**Fig. 5.2**). CB1 activation contributes to the reinforcing properties of drugs and modulates drug self-administration [for review, see 364] and appears to be important for striatal plasticity. CB1 activation is involved in the long-term synaptic depression (LTD) both in the AcbC and the DLS [365, 366] that is impaired in rats vulnerable to cocaine addiction [195].

Within the dorsal striatum, the CB1 expression pattern follows a mediolateral gradient resulting in a greater expression in the DLS [367] in which CB1 modulates the balance between goal-directed and habitual actions. Disruption of cortico-striatal eCB activity gates habit formation [368] and CB1 activity has been shown to be necessary for habitual learning [369]. The roles of endocannabinoid system in habit formation seems to be functionally opposing to the endogenous opioid system.

b. Endogenous opioid system

Endogenous opioid peptides are processed from three precursors: Proopiomelanocortin, proenkephalin and prodynorphin which generate several peptides including β-endorphin, met- and leu-enkephalin, dynorphin and neoendorphin, respectively [370]. These peptides show specific affinity for the three opioid receptors, i.e. dynorphins, enkephalins and β-endorphins display a greater affinity for κ-, δ- and μ-opioid receptors (κ-OR, δ-OR and μ-OR, all Gi/0-protein-coupled), respectively [371-373]. They are all express within the striatum and the mesolimbic

system where they play a pivotal role in different aspects of reinforcement, rewards and addiction [374]. Pharmacological and genetic modulation of opiate receptors have revealed that the endogenous opioid system is predominantly involved in the reinforcing properties of addictive drugs, the tolerance to opiates and the physical dependence to various drugs of abuse [for review, see 375]. Nevertheless, the different opioid peptides and their associated receptors do not show the same modulatory effects on these behavioural aspects. Interestingly, dynorphin/κ-opioid receptors, which are strongly expressed in the AcbC, (κ-ORs being expressed both on dopaminergic and glutamatergic terminals [376, 377]), control the activity of mesolimbic dopaminergic neurons [378] by inhibiting neurotransmitter release, thereby diminishing the reinforcing properties of drugs of abuse [379] (**Fig. 5.2**).

The opioid system is also necessary for instrumental learning where it gates goal-directed actions and its disruption by administration of Naloxone during learning enhances habit formation [380]. More importantly, beyond their contribution to the reinforcing properties of addictive drugs, μ-ORs have been recently involved in their incentive properties, and to mediate the development and expression of incentive habits for heroin, as well as cocaine and alcohol [260, 381].

c. *Adenosinergic system*

Aside being a critical component of the cellular physiology, adenosine also plays an important role in fine regulation of neuronal activity. Adenosine is derived intracellularly and extracellularly from adenosine monophosphate (AMP) and various enzymatic reactions [382-384]. Four types of metabotropic adenosine receptors have been identified within the central nervous system: A1, A2A, A2B and A3 which are either coupled to “inhibitory” Gi/0- / Gq- proteins (A1 and A3), or “stimulatory” Gs / Golf -proteins (A2A and A2B) [385, 386]. A1 and A2A are both highly expressed in the striatum and they are the main adenosine receptors involved in the addictive properties of drugs of abuse. A2A and A1, expressed on postsynaptic medium spiny neurons, decrease the affinity of dopamine for its receptors by forming A2A-D2 and A1-D1 receptors heteromers [387-390] (**Fig. 5.2**). In addition, A1 stimulation reduces neurotransmitter release when expressed on dopaminergic terminals [391]. A2A and A1, together, can form heterodimers on glutamatergic terminals where they either facilitate or inhibit glutamate release depending on the synaptic

concentration of adenosine [390, 392-394].

A2A also modulates glutamatergic neurotransmission by forming heteromers with CB1 receptors [395, 396]. Both genetic and pharmacological studies targeting A1 and A2A receptors have demonstrated a contribution of the adenosinergic system in the reinforcing properties of various drugs of abuse [for review, see 397].

Adenosinergic signalling in the striatum has been also shown to be necessary for habit formation [399] and in contrast, for the acquisition of goal-directed behaviour [400].

Overall, endocannabinoids, endogenous opiates and the adenosinergic system seem all to be lying at the crossroads of drug reinforcement, regulation of the control over instrumental responding by A-O and S-R associations and striatal synaptic plasticity mechanisms. Therefore, they represent perfect candidate systems to be investigated in the context of the intrastriatal functional shifts associated with the development of drug seeking habits and compulsivity.

Materials and methods

Brains of rats trained to self-administer either heroin (n = 69) or cocaine (n = 41), the behavioural characterisation of which was described in chapter 3, were harvested (freshly frozen) 45 min after the last behavioural session. Brains were processed into coronal sections either for in situ hybridisation or micro punching for qPCR assays (**Fig. 5.3**). The segregation in two independent populations for *in situ* hybridisation and qPCR was designed such that the two groups displayed the same behavioural profile, including the same total number of active lever responses both overall and during the first drug-free interval of the daily sessions throughout their self-administration history. The population used in the qPCR experiment was also selected to display a pattern of responding during the first interval of the punished sessions and a number of shocks received during these punished sessions that matched those observed in the cocaine group so as to be able to compare the cellular correlates of compulsive heroin and compulsive cocaine seeking behaviour.

The delay between the end of the session and brain harvesting was implemented to assess mRNA levels of the immediate early gene ZIF-268, which like all the transcription factors belonging to this family shows a transient increase in mRNA levels 45-60 min following cell

activation [401-403].

Thus, *in situ* hybridisation was conducted on serial coronal sections of the brains from rats trained to self-administer heroin under SOR (n = 23) or FI15 (n = 23). qPCR was carried out on the cDNA libraries prepared from the micro-punches of the brains from rats trained under a SOR for either heroin (n = 23) or cocaine (n = 30) or a cocaine FI-15 schedule of reinforcement for cocaine (see Chapter 3).

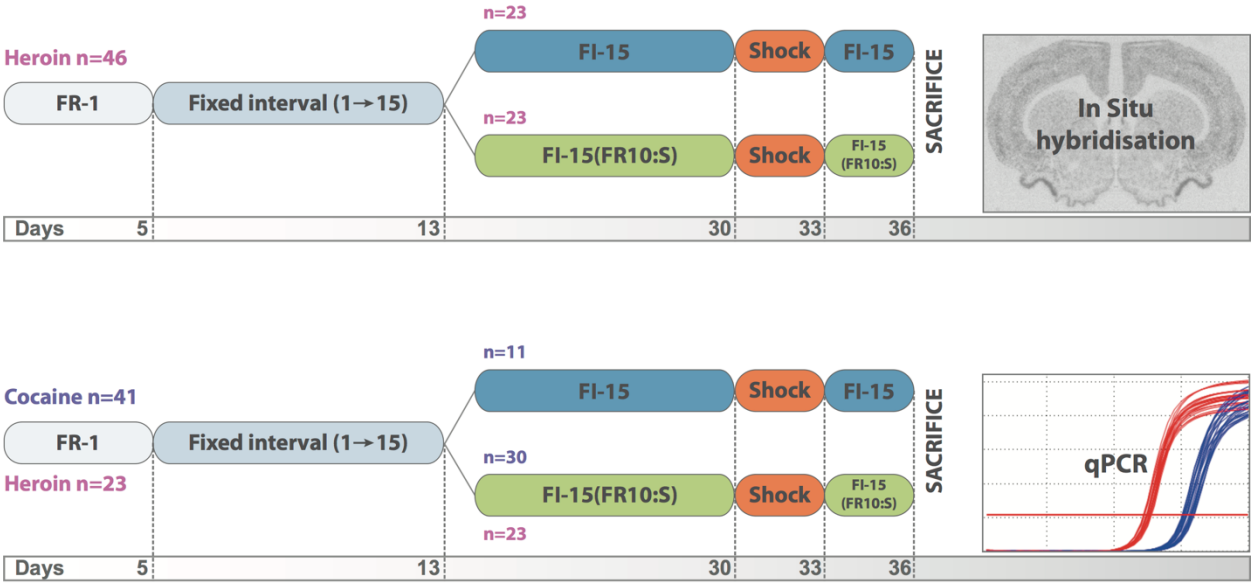


Figure 5.3: Schematic representation of the experimental design and timeline. *Rats were trained to self-administer heroin (n = 69) or cocaine (n = 41) under the protocoles described in Chapter 3. Following sacrifice, brains were harvested and prepared either for in situ hybridisation (heroin: SOR (n = 23) and FI-15 (n = 23) or qPCR (heroin: SOR (n = 23), cocaine: SOR (n = 30) and FI-15 (n = 11)).*

Each individual population therefore consisted of individuals with different compulsive profiles, as characterised by the number of shocks received during the last two shock sessions (see chapter 3). For the *in situ* hybridisation experiment, the segregation into three non-overlapping populations (namely HC, IC and LC) was carried out by defining a quota of HC rats around 20% (as described in Chapter 3). For the qPCR experiment the segregation of the SOR groups in two non-overlapping populations (namely HC and LC) was carried out following the criteria described in Chapter 3 in order to identify HC subjects that received at least 3 or more shocks as opposed to LC rats that received 2 or less shocks. The FI-15 group (cocaine group), used here as a control, was too small to be subdivided into subpopulations.

1. In situ hybridisation

mRNA levels of ZIF268 (also called "early growth response 1", Egr1) were measured throughout the corticostriatal circuitry in a hotspot detection using radioactive *in situ* hybridisation, as described in chapter 2. The oligonucleotide probe used specifically to target ZIF268 mRNA was designed using online tools available at the national centre for biotechnology information's website.

a. Probe design

Oligonucleotide probes were preferred to RNA probes because of their relatively higher resistance to degradation and their small size (40-60 nucleotides) which allows an advantageous tissue penetration (increasing the sensitivity of signal detection) while maintaining a very high specificity to the target mRNA sequence. Several characteristics should be taken in consideration prior to designing an oligonucleotide probe, i.e. the specificity of the sequence to the target mRNA, its size and the percentage of Guanine (G) and Cytosine (C) contained within the sequence. The GC percentage is crucial to calculate the melting temperature (Tm), using the Wallace equation [404], which directly influences the temperature at which the hybridisation is carried-out.

The ZIF268 probe sequence (*5'-CTGTGTGCAGGAGACGGGTAGGTAGAGGAGCCCGG AGAGGAGTAA*) contained 45 nucleotides, with a 60% GC content which conferred a Tm of 74.55ºC. The hybridisation buffer contained 50% Formamide which decreases the Tm by 32.5ºC (~0.65ºC/%,[405]) allowing the hybridisation to be conducted at 42ºC, in conditions that do not damage the brain tissue.

The probe was specific to ZIF-268 mRNA with which it displayed a 100% homology to the sequence 1314-1358 of mRNA, as confirmed by nucleotide BLAST (Basic Local Alignment Search Tool) (**Fig. 5.4**).

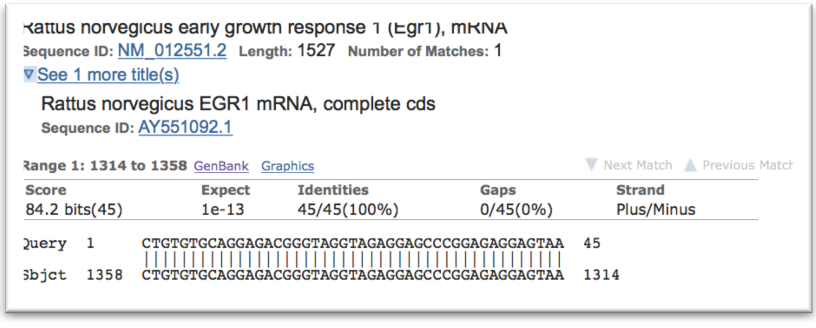


Figure 5.4: Screenshot of a BLAST outcome showing the specificity of the sequence of the ZIF-268 oligonucleotide probe. *The probe sequence was aligned to the mRNA bank from the NCBI. The probe showed a 100% sequence homology with ZIF-268 mRNA of rattus norvegicus. No sequence homologies were found for other mRNA from this species.*

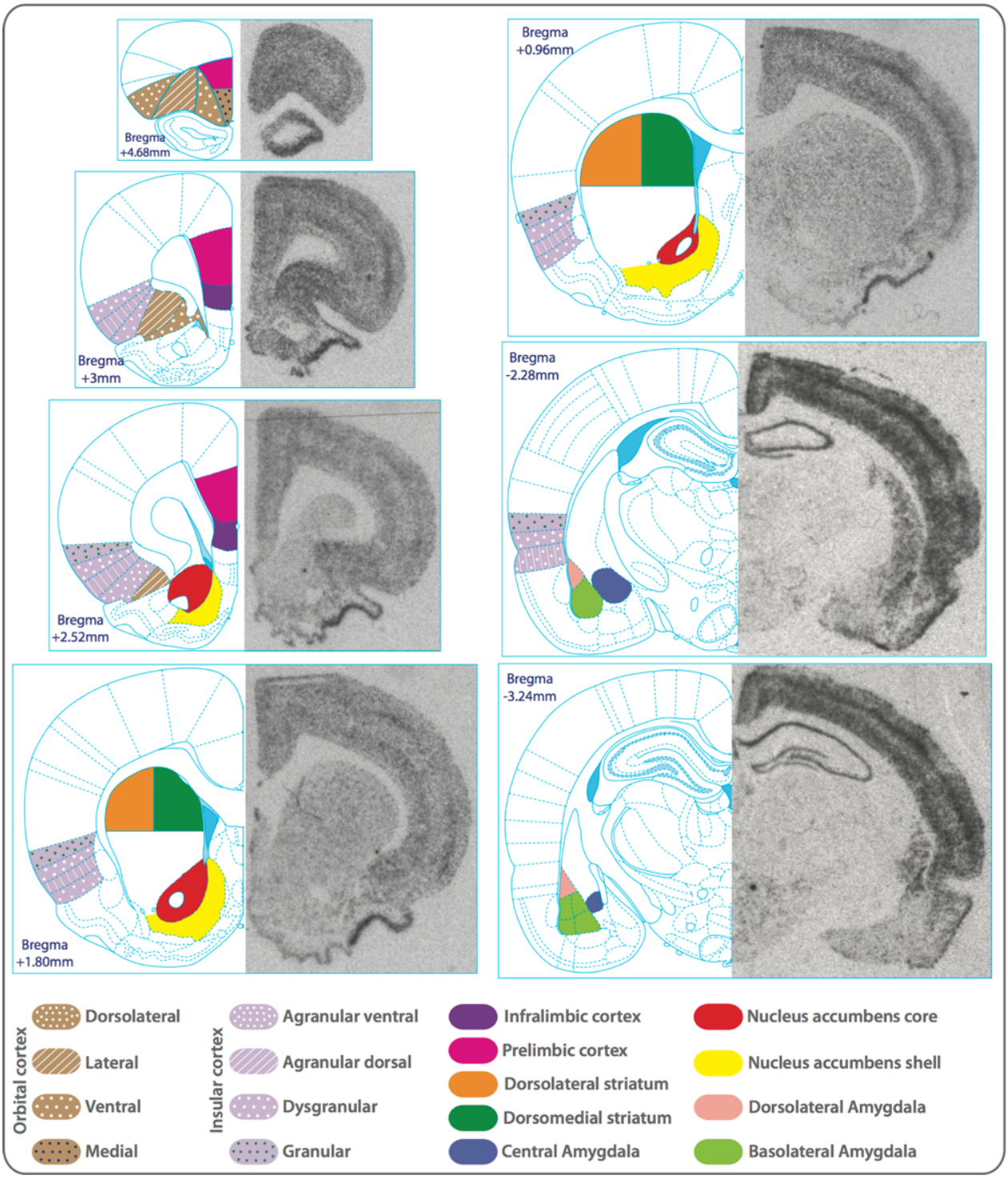


Figure 5.5: Schematic representation the structures of the corticostriatal cicuitry investigated with in situ hybridisation hotspot analysis*. The right panel of each brain slice is a picture of the in situ hybridisation film showing the nature and quality of the dignal obtained in the detection of ZIF-268 mRNA. The left panels represent the delineation of the investigated brain structures [from 242].*

The oligonucleotide probe was synthesised by Sigma-Aldrich® using a manufacturing process that ensures high purity.

The various steps of the *in situ* hybridisation procedure, i.e. probe labelling, pre-hybridisation tissue treatment, probe hybridisation, post-hybridisation treatment and signal acquisition were conducted as described in chapter 2.

b. Quantification

The hotspot analysis performed by *in situ* hybridisation against Zif268 mRNA required investigation of multiple structures throughout the brain. Thus, brains were processed into in 12µm coronal sections through the anteroposterior axis from Bregma +5.2mm to Bregma -4.52mm according to the rat brain atlas [406]. ZIF268 mRNA levels were obtained by measuring the optical density values of the brain structures schematically shown in **Fig. 5.5** to which was subtracted the background taken from passing fibres [407]. Quantification was carried out with the help of Ms. Katie Cudmore in the context of a MPhil project.

2. Quantitative polymerase chain reaction

qPCR assays were conducted on cDNA libraries obtained from RNA contained micro-punches of the AcbC, AcbS and aDLS of rats trained to self-administer heroin (n = 23) or cocaine (n = 30) in the compulsive habitual drug seeking behaviour model detailed in chapter 3. Rats (n = 11) trained under a FI-15 schedule of reinforcement for cocaine were also included in this study.

The couples of primers used were purchased from Qiagen® (*RT^2 qPCR Primer assay,* cat no: 330001) and designed by the company to be highly compatible with the *RT^2 SYBR Green Mastermix* (*Qiagen*) used for the polymerase chain reaction step as described in chapter 2. Qiagen® ensures that these primers were designed and manufactured providing uniform PCR efficiency and amplification conditions. Experimental assays from this company ensured the amplification of a single amplicon of the expected size with a high efficiency. The sequence of each primers remained unknown but their other major characteristics, i.e. uniGene number, band size, RefSeq accession number and position of the amplicon in the RefSeq sequence were available and are show in **Table. 5.1**.

Target gene	Qiagen® Ref	Gene symbol	UniGene n°	Band size (bp)	RefSeq Accession	Ref position
Dopamine D1 Rc	PPR06790A	Drd1	Rn.24039	82	NM_012546.3	555
Dopamine D2 Rc	PPR06827A	Drd2	Rn.87299	85	NM_012547.1	1506
Dopamine transporter	PPR44664C	Slc6a3	Rn.10093	80	NM_012694.2	1460
mu-opioid Rc	PPR06761A	Oprm1	Rn.10118	151	NM_013071.2	866
kappa-opioid Rc	PPR06833G	Oprk1	Rn.89571	73	NM_017167.2	630
Adenosine A1 Rc	PPR48482A	Adora1	Rn.32078	188	NM_017155.2	612
Adenosine A2A Rc	PPR45279F	Adora2a	Rn.11180	132	NM_053294.4	1539
Pro-dynorphin	PPR49782A	Pdyn	Rn.44471	180	NM_019374.3	603
cyclophilin A	PPR06504A	Ppia	Rn.1463	89	NM_017101.1	486

Table.5.1: Characteristics of the couples of primers purchased for the qPCR assays

The "quantitative" nature of the qPCR approach is based on the measure of the expression of a specific transcript of interest relative to a house keeping gene, used as an internal control, thereby enabling comparison of the relative expression of the target mRNA across experimental groups or conditions. The house-keeping gene used in this study was Cyclophilin A (also known as peptidylprolyl isomerase A), the gene product of which is an ubiquitous enzyme involved in protein folding, trafficking and assembly processes [408]). Cyclophilin was selected A was selected because its mRNA levels have been shown to be impervious to drug exposure.

Potential differences in the Cyclophillin A mRNA (actual Ct values) were systematically investigated between each individual data point and comparisons between groups or structures were not conducted if these Ct values were found to be statistically different.

Samples were loaded on 96-well plates (as described in chapter 2) according to a plate design whereby the samples from groups (SOR heroin, SOR cocaine and FI-15 cocaine) and subgroups (HC and LC) were distributed on two plates each containing similar number of samples from each group/subgroup. This experimental design enabled the quantification of 2 target mRNAs per assay by comparing their Ct to the one of one adjacent Cyclophillin A mRNA. Therefore, for each tandem of target mRNA (D1 Rc/D2 Rc, DAT/A2A Rc, kappa Rc/dyn, mu Rc/A1 Rc) a single Cyclophillin A Ct value was used.

The different steps of the qPCR assays, i.e. RNA extraction, reverse transcription, PCR and data analysis were conducted as described in chapter 2.

Results

1. Behavioural characterisation of the experimental populations

A cohort of rats was trained to self-administer heroin under SOR and tested for individual sensitivity to punishment, as described in Chapter 3. It was subsequently split in two populations (destined to *in situ* hybridisation and qPCR, respectively) that were perfectly matched for the behaviour they displayed over training, as illustrated in **Fig. 5.6**.

Thus, no differences were observed between rats that were used for in situ hybridisation (ISH group) and those used for qPCR in the overall heroin seeking responses displayed throughout the self-administration sessions *[main effect of group: $F(1,44) < 1$; session: $F(35,1540) = 49.669$, $p < 0.001$; and group x session interaction: $F(35,1540) < 1$]* (**Fig. 5.6A**). The two groups did not differ either in their level of responding during the first heroin-free interval throughout the self-administration sessions *[main effect of group: $F(1,44) < 1$; session: $F(25,1100) = 25.063$, $p < 0.001$; and group x session interaction: $F(25,1100) = 1.5783$, $P < 0.05$]* (**Fig. 5.6B**). Despite this statistically significant group x session interaction, likely attributable to the slight differences in the behavioural adaptations to punishment, post-hoc analyses confirmed that the two groups did not differed on any single day.

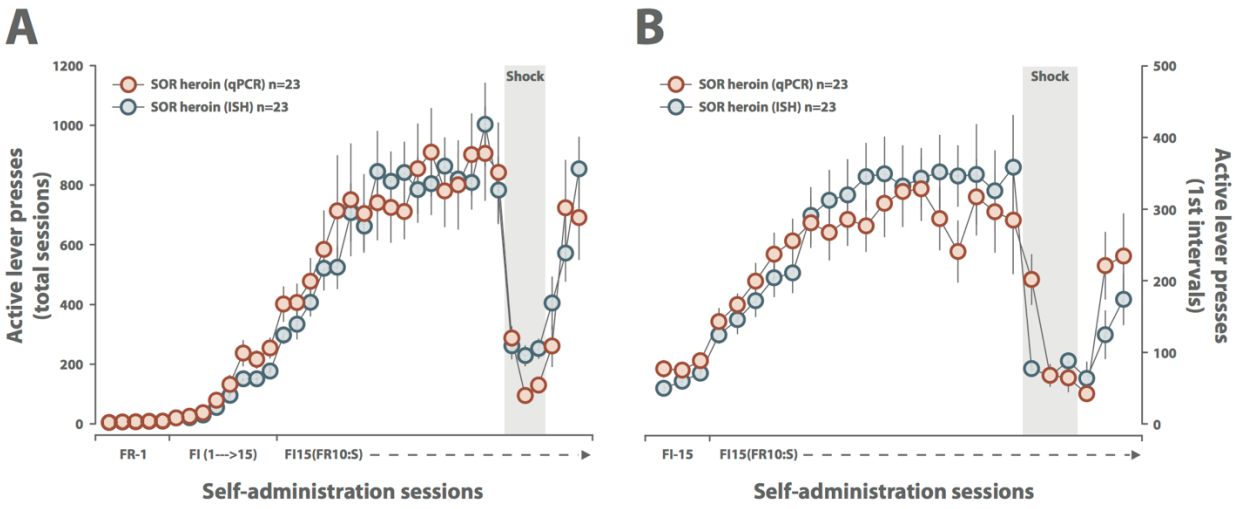


Figure 5.6: behavioural characterisation of the sub-populations subsequently used in in situ hybridisation (ISH) and qPCR experiments. *No difference was observed between experimental populations in the number of active lever presses expressed during daily sessions* ***(A)*** *or the first drug free interval of FI15 and SOR* ***(B)****.*

As predicted, HC identified in the SOR or FI15 ISH sub-populations received many more punishment than LC rats over the last two punished sessions *[main effect of group for SOR: F(1,12) = 42.302, p < 0.001 and FI-15: F(1,15) = 97.514, p < 0.001]*. HC rats also displayed higher level of responding than LC rats during the punished first 15 min intervals *[SOR groups: main effect of group: F(1,12) = 29.744, p < 0.001; session: F(2,24) = 2.4675, p > 0.1; and group x session interaction: F(2,24) < 1; FI-15 groups: main effect of group: F(1,15) = 61.881, p < 0.001; session: F(2,30) = 5.4122, p < 0.01; and group x session interaction: F(2,30) = 9.5158, P < 0.001]* (**Fig. 5.7**).

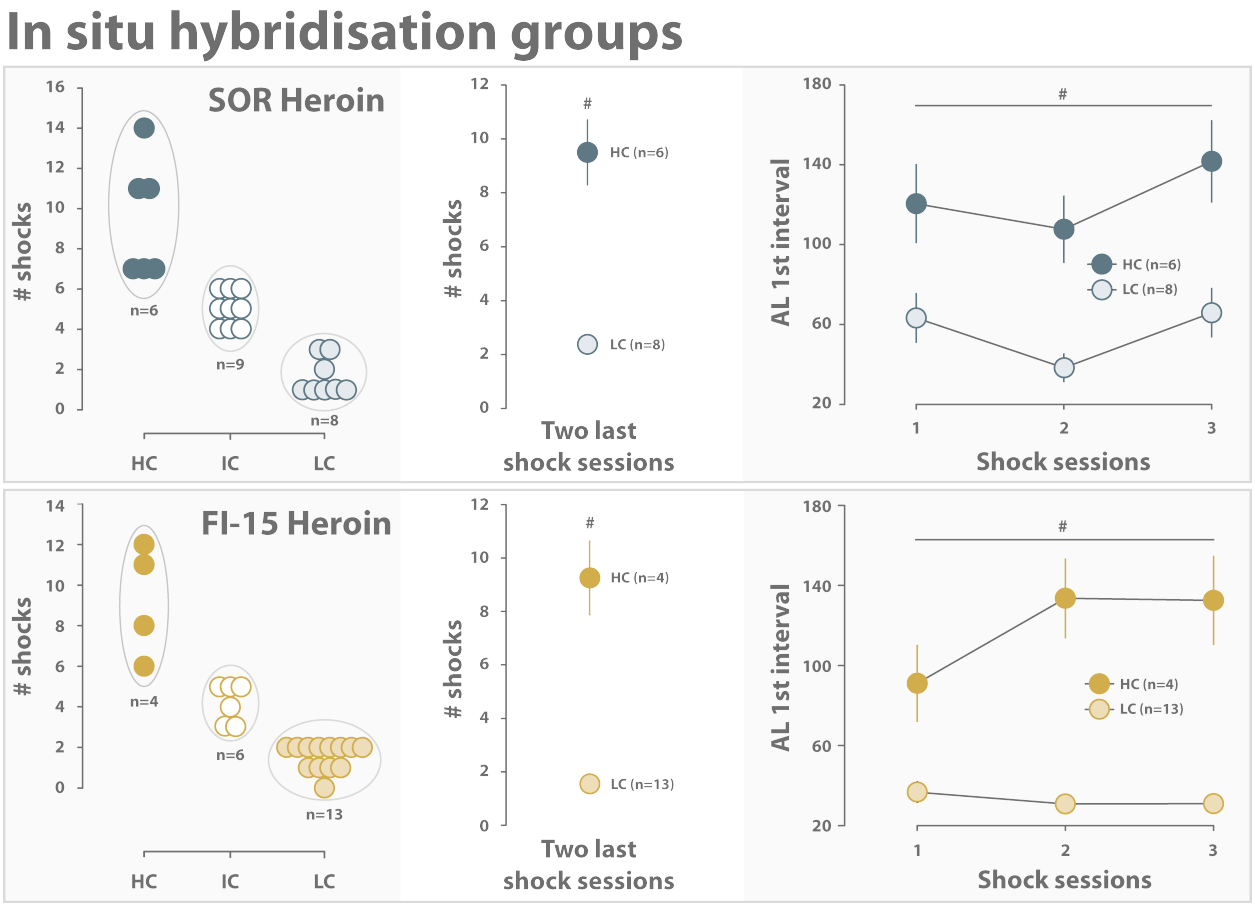


Figure 5.7: Characterisation of inter-individual differences in compulsive heroin seeking in the experimental populations subsequently used for in situ hybridisation. *Both SOR and FI-15 conditions (respectively top and bottom panels) contained individuals displaying HC, IC and LC profiles. In both conditions, when exposed to punhsiment, HC rats received more shocks, and displayed a higher level of responding during the first, drug-free interval, than LC rats [* : p<0.001].*

Similarly, HC rats identified in the SOR heroin and SOR cocaine subpopulations, subsequently used for qPCR experiments, received more shocks than LC counterparts during the last two punished sessions *[main effect of group for the SOR heroin: F(1,21) = 50.565, p < 0.001 and the SOR cocaine group: F(1,28) = 23.040, p < 0.001]*. HC rats also displayed higher level of responding

than LC rats during the punished first 15 min intervals *[SOR heroin groups: main effect of group: F(1,21) = 9.9038, p < 0.01; session: F(2,42) = 20.366, p < 0.001 ; and group x session interaction: F(2,42) < 1 ; SOR cocaine groups: main effect of group: F(1,28) = 10.782, p < 0.01; session: F(2,56) = 1.1632, p > 0.1 ; and group x session interaction: F(2,56) = 3.9339, P < 0.05]* (**Fig. 5.8**).

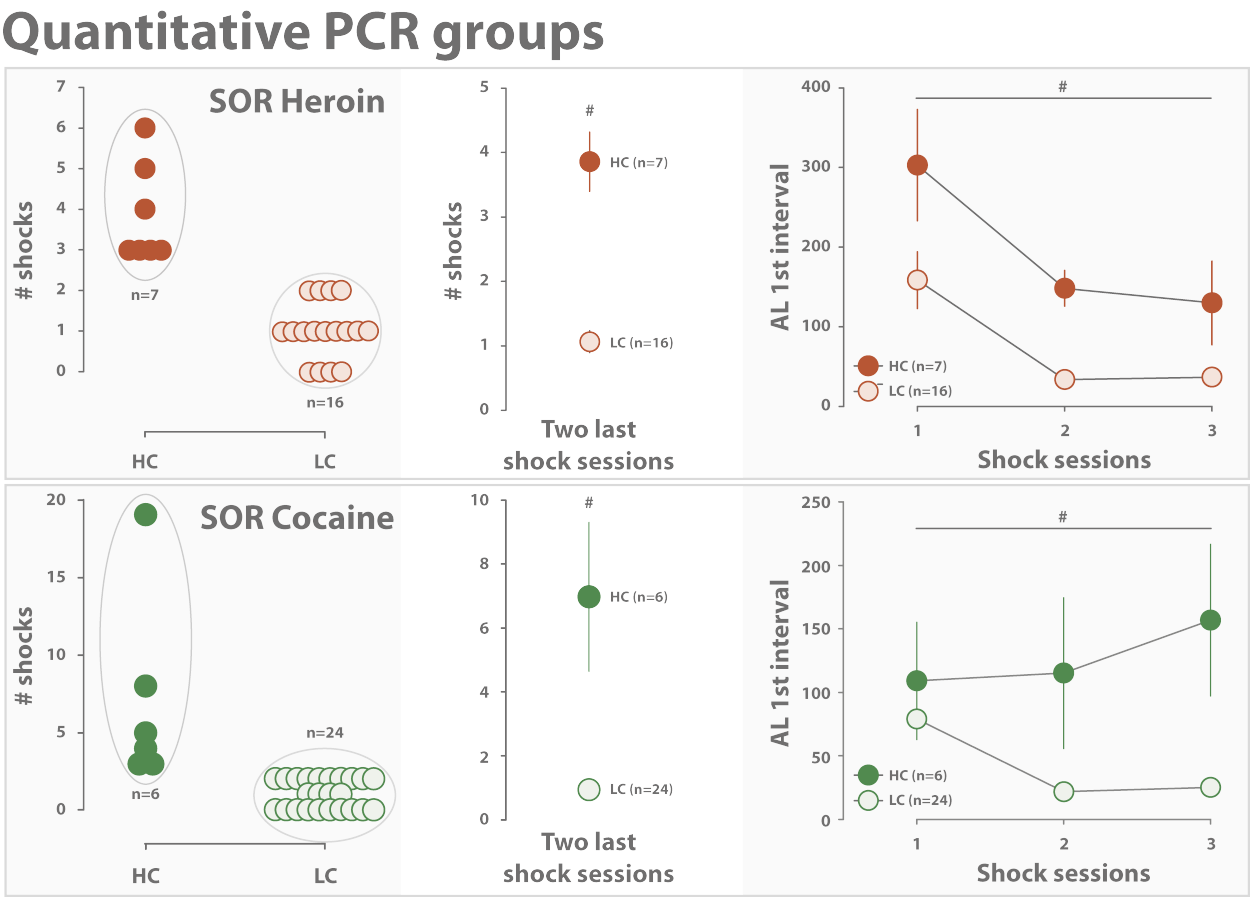


Figure 5.8: Characterisation of inter-individual differences in compulsive heroin seeking in the experimental populations subsequently used for qPCR. *Both SOR and FI-15 conditions (respectively top and bottom panels) contained individuals displaying HC, IC and LC profiles. In both conditions, when exposed to punhsiment, HC rats received more shocks, and displayed a higher level of responding during the first, drug-free intervals, than LC rats [* : p<0.01].*

The selection of compulsive and non-compulsive rats in the SOR heroin condition was carried-out so as to match the levels of compulsivity to those observed in the SOR cocaine group, in terms of both electric foot-shocks received during the three punished sessions *[HC rats: main effect of drug: F(1,11) < 1 ; session: F(2,22) = 1.6637, p > 0.1; and drug x session interaction: F(2,22) = 1.1508, p > 0.1; LC rats: main effect of drug: F(1,38) < 1, p < 0.01 ; session: F(2,76) = 36.682, p < 0.001 ; and group x session interaction: F(2,76) < 1]* (**Fig. 5.9A**), and active drug seeking level during these punished sessions [*HC rats: main effect of drug: F(1,11) = 1.0562, p>0.1; session: F(2,22) = 2.6615, p>0.05; and group x session interaction: F(2,22) = 5.4401, p < 0.05; LC groups:*

main effect of group: F(1,38) = 5.1668, p < 0.05; session: F(2,76) = 27.183, p < 0.001; and drug x session interaction: F(2,76) = 3.8997, P < 0.05]. In spite of the drug x session interaction reported here, post hoc analysis revealed that LC rats in the two drug conditions only differed during the first punished session (**Fig. 5.9B**).

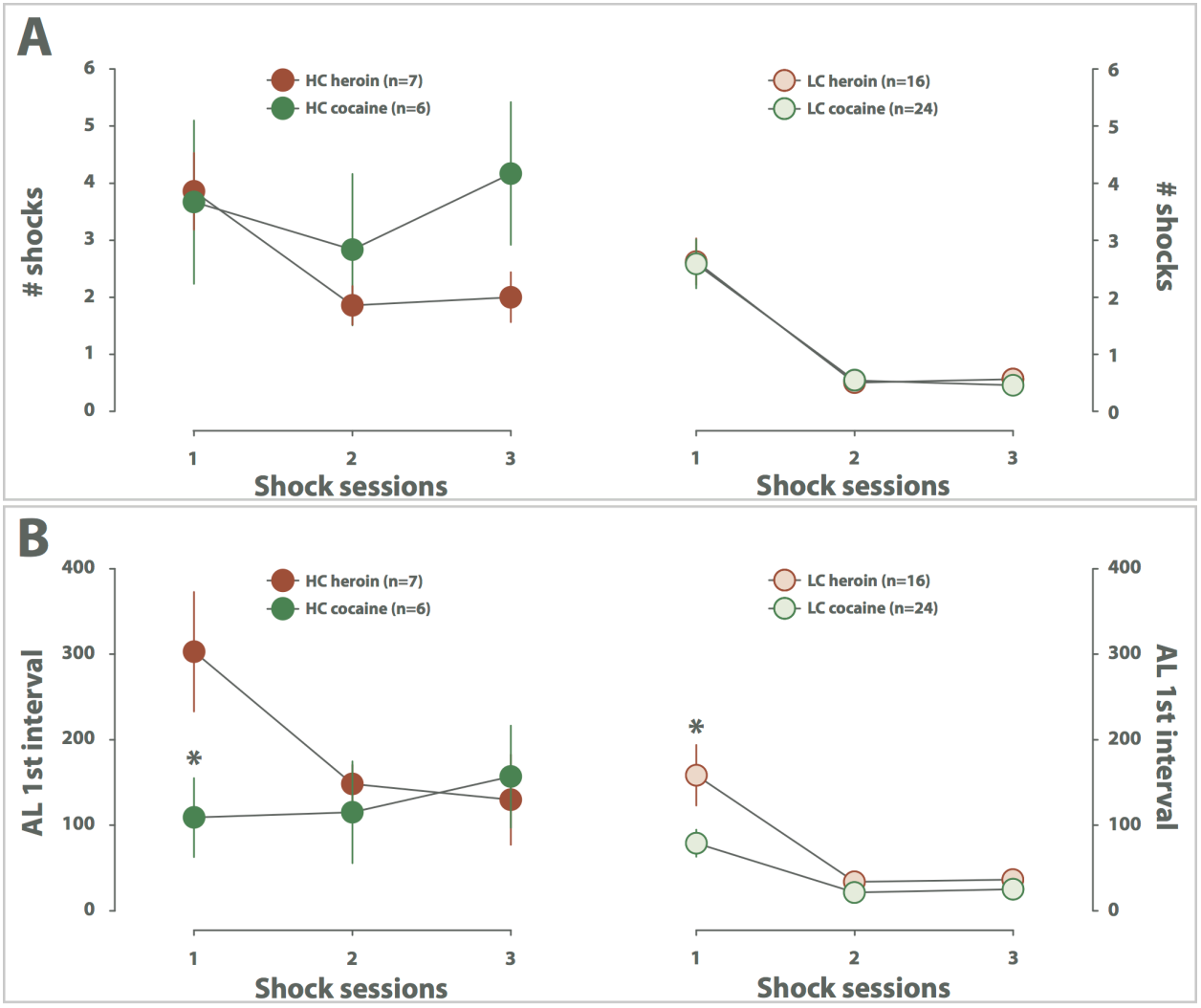


Figure 5.9: Characterisation of the nature of the compulsive behaviour and resilience, respectively, of HC and LC rats subsequently used for qPCR experiments. *The analysis of the number of shocks received (**A**), and level of drug seeking diplayed during the first, drug-free interval (**B**) by HC rats during the punished sessions revealed that the compulsive nature of their heroin seeking was similar between cocaine and heroin. Similarly, LC rats, at the exception of the first punisment session, displayed similar resilience to compulsive heorin and cocaine seeking.*

2. Differential plasticity characterising incentive habits

The quantification of the mRNA levels of the immediate early gene zif268 in the various structures of the corticostriatal circuitry of the brain of both compulsive and non-compulsive rats, with or without incentive habits, yielded a wealth of data, the analysis of which revealed hotspots specifically associated with incentive habits and/or compulsive heroin or cocaine seeking. The means ± sem of the OD, that reflects zif268 mRNA level, obtained in each structure for each condition (SOR versus FI-15) and subgroups (HC versus LC) are presented in **Table 5.2**.

a. Intrastriatal adaptations

The hotspot analysis throughout the striatum revealed differential recruitment of striatal territories in rats in the SOR group as compared to the FI15 group. Thus, incentive habits (displayed by rats exposed to SOR as compared to FI15) were associated with an increased plasticity in the aDLS *[main effect of group: $F(1,41) = 4.0887$, $p < 0.05$]* as well as medial Acb shell (mAcbS) *[main effect of group: $F(1,43) = 4.2765$, $p < 0.05$]* (**Fig. 5.10A&D**), but not in the aDMS, the lateral Acb shell (lAcbS) or even the AcbC *[group effect: $F(1,42) < 1$]* (**Fig. 5.10B**, see **Table 5.3**).

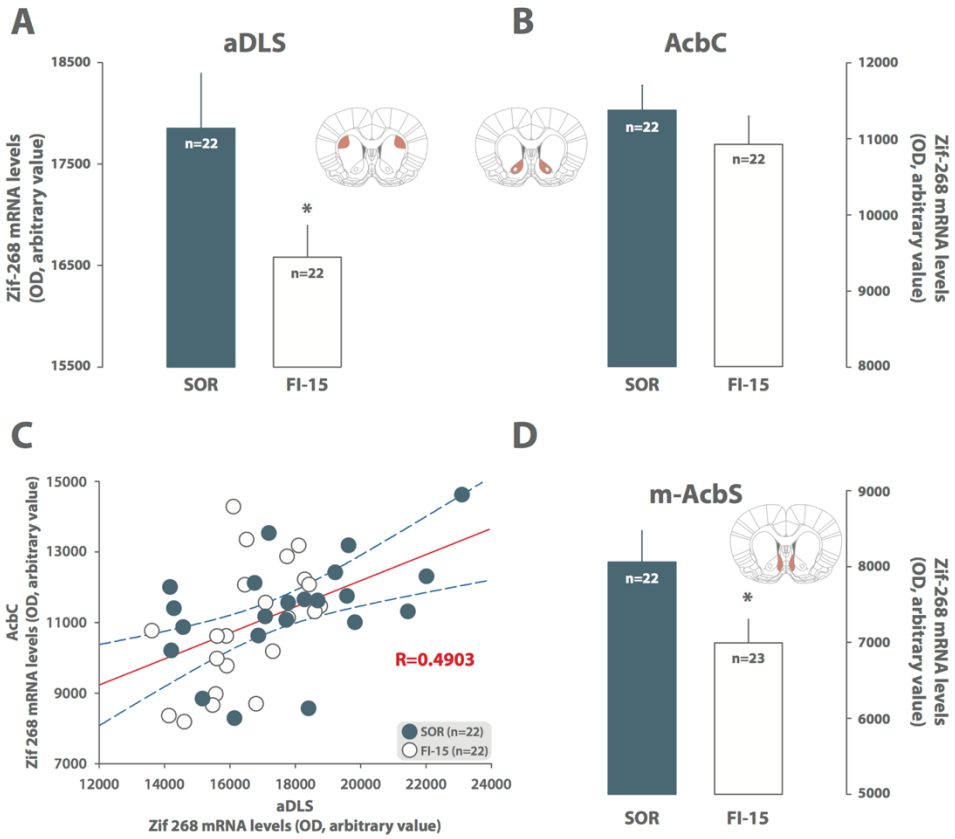


***Figure 5.10: ZIF-268 mRNA levels in the striatal areas of rats trained to self-administer heroin under a SOR or FI-15 schedule of reinforcment**. Rats with an history of SOR displayed higher levels of zif268 mRNA than those with an history of FI15 in the aDLS **(A)** and the medial shell (m-AcbS) **(D)**. However no difference in zif268 mRNA was observed in the AcbC **(B)** even if the level of zif268 measured in the AcbC correlates with the one measured in the aDLS in population-wide dimensional analyses **(C)**. [*: $p < 0.05$]*

	Overall population			SOR			FI-15			HC - SOR			LC - SOR			HC - FI-15			LC - FI-15		
	n	mean	sem	n	mean	sem	n	mean	sem	n	mean	sem	n	mean	sem	n	mean	sem	n	mean	sem
PL	46	24862.70	1032.95	23	26491.32	1266.67	23	23234.08	1587.26	6	25727.81	2656.11	8	23670.16	2274.33	4	26779.79	3763.79	13	23244.95	2362.00
IL	45	22437.60	948.69	23	24680.06	1171.90	22	20093.20	1356.83	6	23479.95	2080.30	8	22857.49	2472.60	4	21694.15	3420.00	13	19568.46	2050.85
mOFC	44	24376.84	1507.90	21	24657.52	2263.25	23	24120.56	2060.09	6	21691.57	4938.36	7	22618.44	4552.63	4	32194.05	4566.50	13	23255.04	2925.69
vOFC	45	32327.78	1220.06	23	33753.86	1547.10	22	30836.89	1884.38	6	31943.29	2896.47	8	32181.72	3453.84	4	36389.40	3852.96	13	29531.68	2698.44
lOFC	45	26212.72	1296.32	23	28106.54	1770.37	22	24232.81	1845.00	6	24728.56	3037.84	8	26316.62	3689.55	4	30525.99	2601.63	13	22657.36	2690.91
AI cx	45	17343.27	870.17	23	18452.09	1324.19	22	16184.04	1095.18	6	17378.82	1608.84	8	17081.41	2848.19	4	20039.88	1916.02	13	15737.67	1562.10
Di cx	45	13806.67	764.32	23	14722.47	1310.22	22	12849.25	733.55	6	13048.17	1665.74	8	12755.53	2015.41	4	15375.08	996.22	13	12154.37	1037.82
aDLS	44	17215.82	325.22	22	17851.02	543.68	22	16580.62	314.86	6	18176.24	608.49	7	16952.05	1229.80	4	16823.79	577.39	12	16359.21	404.01
aDMS	44	14013.12	343.25	22	14626.68	543.96	22	13399.56	388.26	6	14908.26	704.92	7	14353.84	1029.24	4	13559.30	797.38	12	13134.44	606.43
AcbC	44	11149.38	245.41	22	11374.15	324.60	22	10924.61	369.41	6	10808.73	537.34	7	11877.18	522.83	4	11422.61	1187.31	12	10771.84	504.36
AcbS Lat	45	5948.24	268.81	22	6421.09	395.43	23	5495.95	347.79	6	7108.14	927.26	7	5887.18	645.99	4	5410.02	880.24	13	5065.47	399.58
AcbS Med	45	7522.20	266.64	22	8066.11	414.07	23	7001.94	310.64	6	7560.94	578.23	7	8377.30	1006.03	4	7354.59	922.00	13	6553.26	425.84
Lat BLA	45	157.5934	3.2709	22	162.7714	4.9545	23	152.6406	4.144	5	162.1012	13.7629	8	156.7853	9.685	4	155.1131	3.2465	13	147.0282	5.8817
BLA	46	46.3639	1.9177	23	47.7709	2.8036	23	44.957	2.6466	6	47.4029	5.9875	8	47.52	4.5819	4	47.0649	6.615	13	42.3923	2.3336
CeA	46	69.5771	3.4121	23	72.0515	5.144	23	67.1027	4.5399	6	76.7826	12.1139	8	75.883	8.3438	4	63.6156	13.5353	13	67.8195	6.5127

Table 5.2: descriptive statistics of ZIF-268 mRNA levels in each of the structures of the corticostriatal circuitry investigated in those rats displaying incentive habits (SOR) or habitual heroin seeking, as well as those identified in each condition as being compulsive (HC) and non compulsive (low compulsive, LC).

However, dimensional analyses revealed at the population level, that ZIF-268 mRNA levels in the aDLS were correlated with those in the AcbC, suggesting a functional convergence of these two striatal structures (**Fig. 5.10C**). This correlation was found significant for both conditions (incentive habits, R = 0.4765 and habits, R = 0.5378).

	SOR Vs FI-15		FI-15 : HC Vs LC		SOR : HC Vs LC	
	F	*p*	F	*p*	F	*p*
PL	2.573	0.116	0.551	0.469	0.347	0.566
IL	**6.582**	**<0.05**	0.260	0.617	0.034	0.857
mOFC	0.031	0.861	2.312	0.149	0.019	0.893
vOFC	1.443	0.236	1.642	0.219	0.003	0.961
lOFC	2.297	0.137	2.346	0.146	0.101	0.757
AI cx	1.726	0.196	2.001	0.178	0.007	0.935
Di cx	1.519	0.225	2.645	0.125	0.011	0.917
aDLS	**4.089**	**<0.05**	0.355	0.561	0.714	0.416
aDMS	3.371	0.073	0.135	0.719	0.184	0.676
AcbC	0.836	0.336	0.352	0.562	2.014	0.184
AcbS Lat	3.101	0.085	0.159	0.695	1.223	0.292
AcbS Med	**4.276**	**<0.05**	0.765	0.395	0.451	0.516
Lat BLA	2.478	0.123	0.543	0.473	0.106	0.751
BLA	0.533	0.469	0.729	0.407	0.001	0.988
CeA	0.520	0.475	0.092	0.766	0.004	0.951

Table.5.3: F and p values obtained of the one-way ANOVAs performed on the ODs reflecting the quantification of ZIF-268 mRNA levels in the investigated structures, where conditions (SOR/FI-15) and subgroups (HC/LC) were used as between-subject factors.

b. Cortical adaptations

In parallel with the differential functional recruitment of aDLS and mAcbS, incentive habits were also characterised by a higher recruitment of zif268 in the IL, which, alongside the aDLS contributes to maintain the control over behaviour by S-R associations.

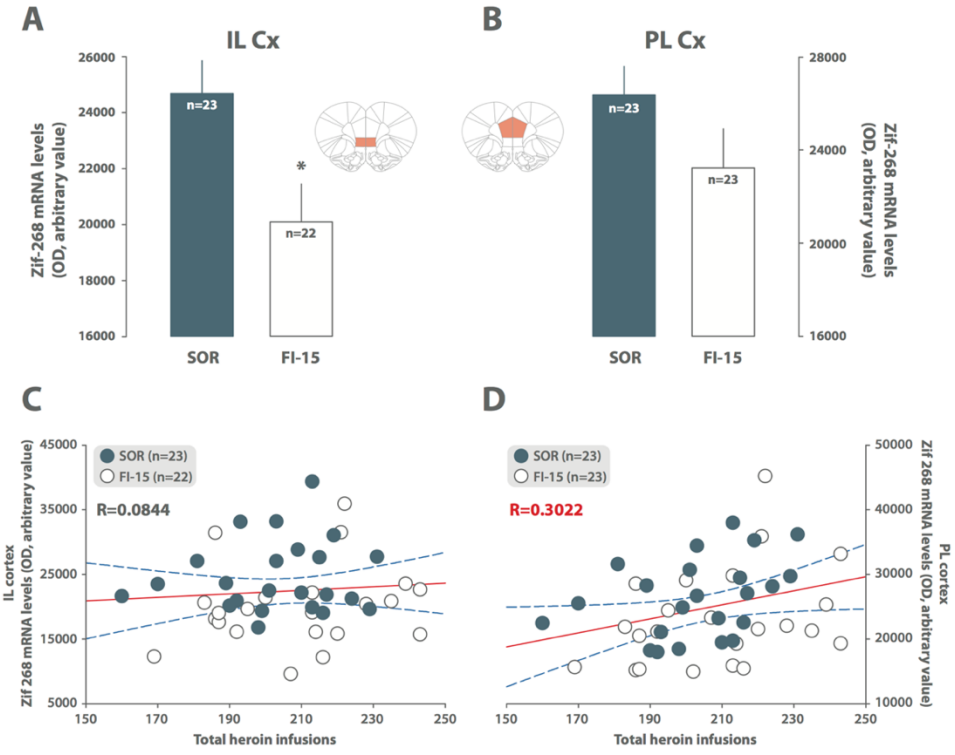


Figure 5.11: ZIF-268 mRNA level in the prefrontal cortical areas of rats with an history of SOR or FI-15 schedule of reinforcement for heroin. *A) rats with an history of SOR displayed higher zif268 mRNA levels in the IL cortex than rats with a history of FI15. B) No difference was observed between the two conditions in the in ZIF-268 levels mearured in the PL cortex. C) At the population level, ZIF-268 mRNA level in the IL cortex did not correlate with the total number of infusions received throughout the heroin SA history in marked contrast with the correlation observed between the total heroin infusions and ZIF-268 mRNA levels in the PL cortex (D). [*: p < 0.05]*

Thus, rats with a history of SOR displayed higher levels of zif268 in the IL cortex as compared to rats with a history of FI-15 *[main effect of group: $F(1,43) = 6.5824$, $p < 0.05$]* (**Fig. 5.11A**).

Surprisingly, whereas a potential decrease in zif268 was expected in the PL cortex, no difference between the two conditions was observed in this structure *[main effect of group: $F(1,44) = 2.5728$, $p > 0.1$]* (**Fig. 5.11B**) as well as all the other cortical areas investigated (see **Table 5.3**).

Irrespective of the conditions under which rats were trained to seek heroin (either SOR or FI15), at the population level, zif268 mRNA levels in the PL cortex, but not the IL cortex, were predicted by the total number of heroin infusions each individual received throughout their drug self-administration history (**Fig. 5.11C&D**). Since these levels of Zif268 mRNA were measured 45 min after a 15 min drug-free seeking challenge, this result suggests an interaction between past history of intoxication and the functional recruitment of the PL cortex during drug seeking.

The recruitment of cellular plasticity in the PL cortex after a seeking challenge, if not different between habits and incentive habits, was also correlated with the magnitude of the recruitment of zif268 in the lateral BLA specifically in the rats with a history of SOR (**Fig.5.12**), suggesting a functional relationship specifically recruited between these structures in the expression of incentive habits.

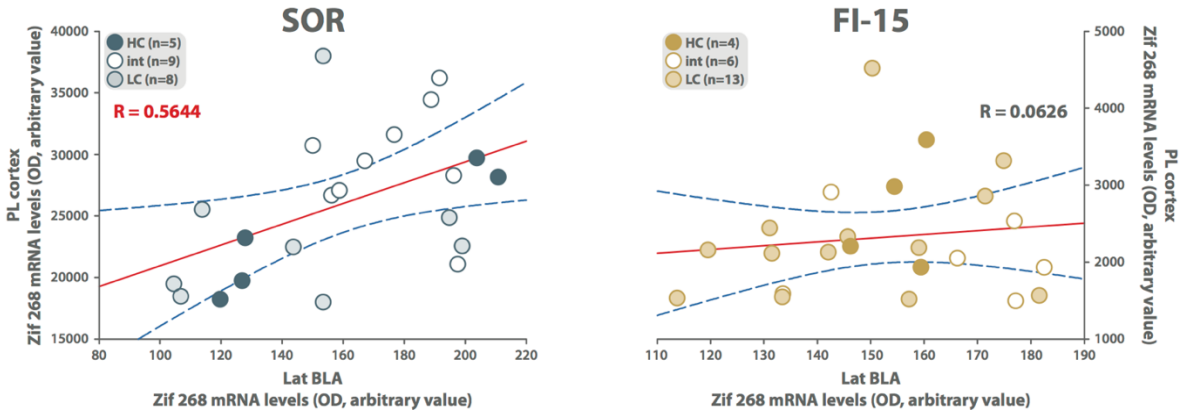


Figure 5.12: Selective relationship between ZIF-268 mRNA levels in the PL cortex and the lateral BLA in incentive habits. *A robust positive correlation between zif268 mRNA levels in the PL cortex and lateral BLA was obersved only in rats with an history of SOR (left), not in rats with an history of FI15 (right).*

c. Amygdalo-striatal adaptations

Within the amygdala, a functional convergence seems to operate in incentive habits between the BLA and the CeA. A robust correlation was observed between the zif268 mRNA levels measured in the BLA and the CeA, but only in rats with an history of SOR (**Fig. 5.13A**).

This convergence in the recruitment of cellular plasticity during the expression of incentive habits observed within the amygdala was extended to the ventral striatal territories to which the BLA send massive glutamatergic projections, namely the AcbC and lat AcbS (**Fig. 5.13B-C**).

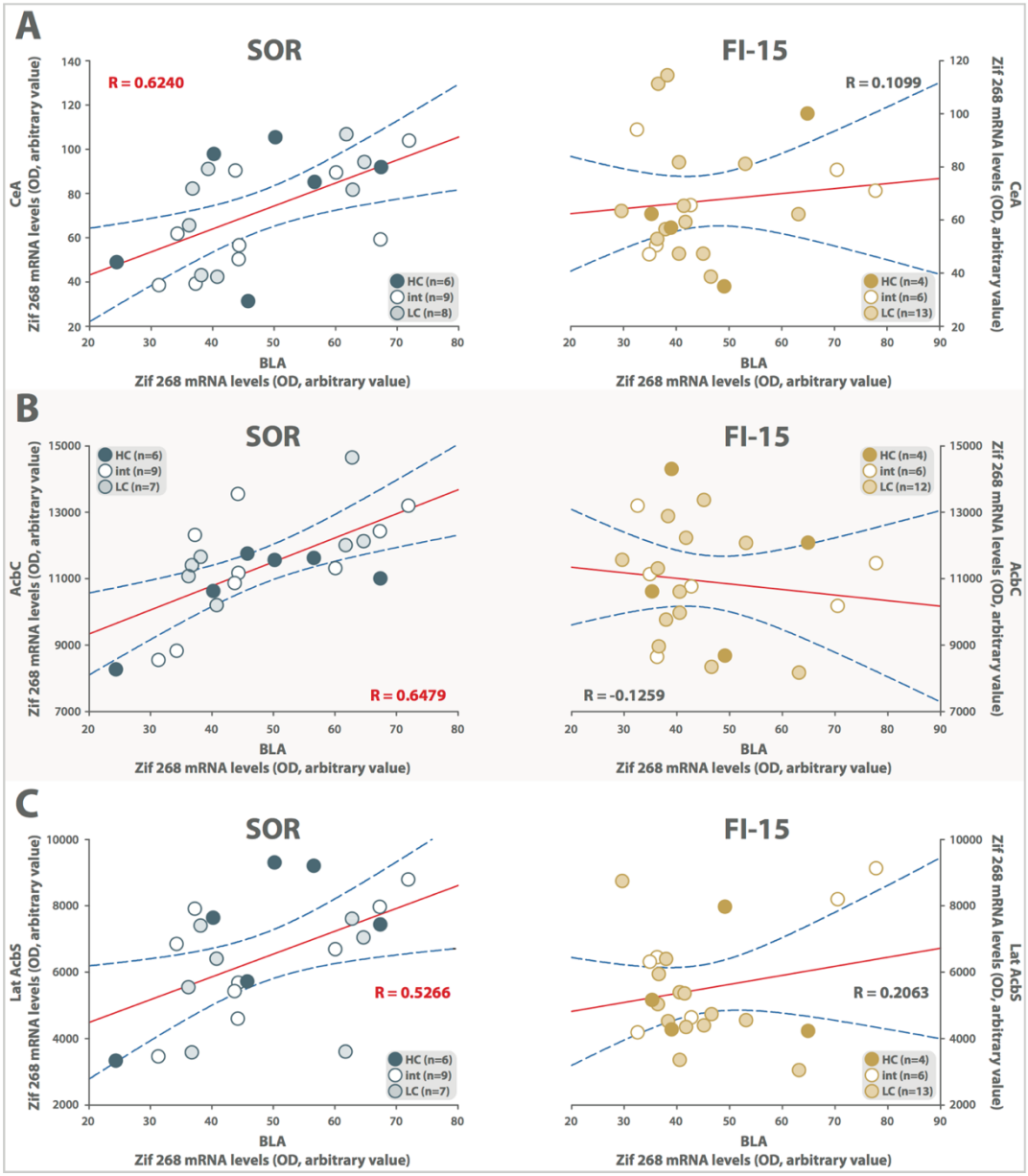


Figure 5.13: Intra-amygdala and amygdalo-striatal functional convergence in incentive habits. *ZIF-268 levels were correlated between BLA and CeA, BLA and AcbC BLA and latAcbS only in the population of rats with an history of SOR.*

Similarly, robust correlations were observed between the zif268 mRNA levels measured in the BLA and the AcbC or the lAcbS, but only in rats with a history of SOR (**Fig. 5.13B&C**).

In marked contrast with the cellular plasticity recruited in the PL during drug seeking, the level of zif268 mRNA in the BLA and the CeA measured following drug-free seeking challenge was predicted by the total number of heroin infusions throughout the self-administration history only in individuals with a SOR training (**Fig. 5.14A&B**). This was not attributable to a differential level of heroin infusions between the two conditions (SOR vs FI15) *[main effect of condition: F(1,44) < 1,* data not shown].

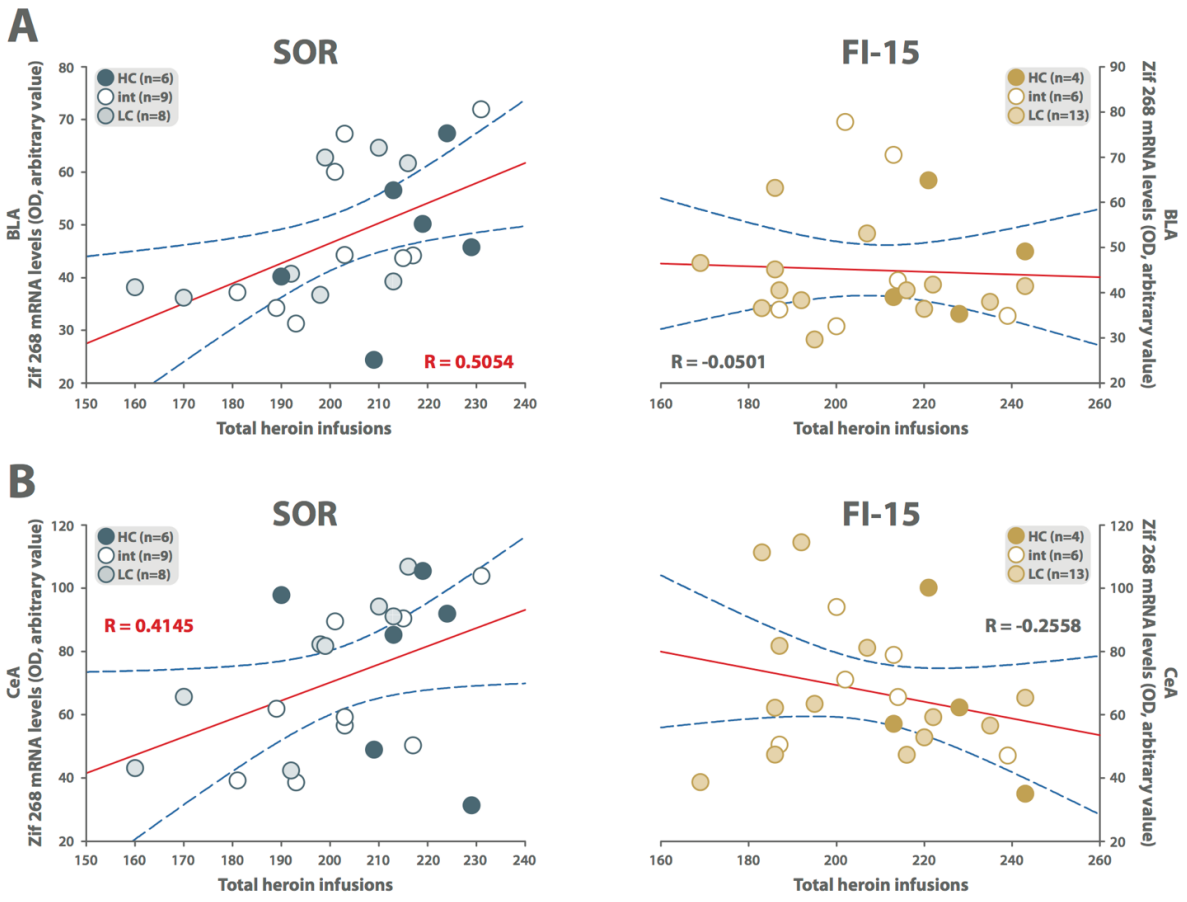


Figure 5.14: BLA and CeA ZIF-268 mRNA level correlates with the total number of heroin infusion received. Correlations were found positively significant only for SOR group, not for FI-15 group.

Besides these aforementioned differential recruitment of cellular plasticity processes within the corticostriatal circuitry no other difference was observed between rats with a history of SOR and those with an history of FI15 (see **Table 5.3**).

d. Hotspot mapping of compulsive heroin seeking behaviour

The between-subject and dimensional analyses carried out on high and low compulsive rats from both the SOR and FI15 conditions revealed that compulsive heroin seeking was not associated with a specific recruitment of cellular plasticity in the structures of the corticostriatal circuitry investigated here. Indeed, no differences were found between HC an LC rats irrespective of their training history, in the zif268 mRNA levels throughout the corticostriatal network (**Table 5.3**).

Nevertheless, the analysis of the differential mRNA levels of the candidate genes within the striatal territories offered insights into cellular mechanisms of compulsive heroin and cocaine seeking behaviour.

3. Striatal correlates of compulsive heroin and cocaine seeking

In order to conduct meaningful direct comparisons in the mRNA levels of candidate genes between HC and LC rats, drugs, conditions or structures, the Ct of the internal standard, namely Cyclophillin A (against which the Cts of each candidate gene are compared), must be similar across.

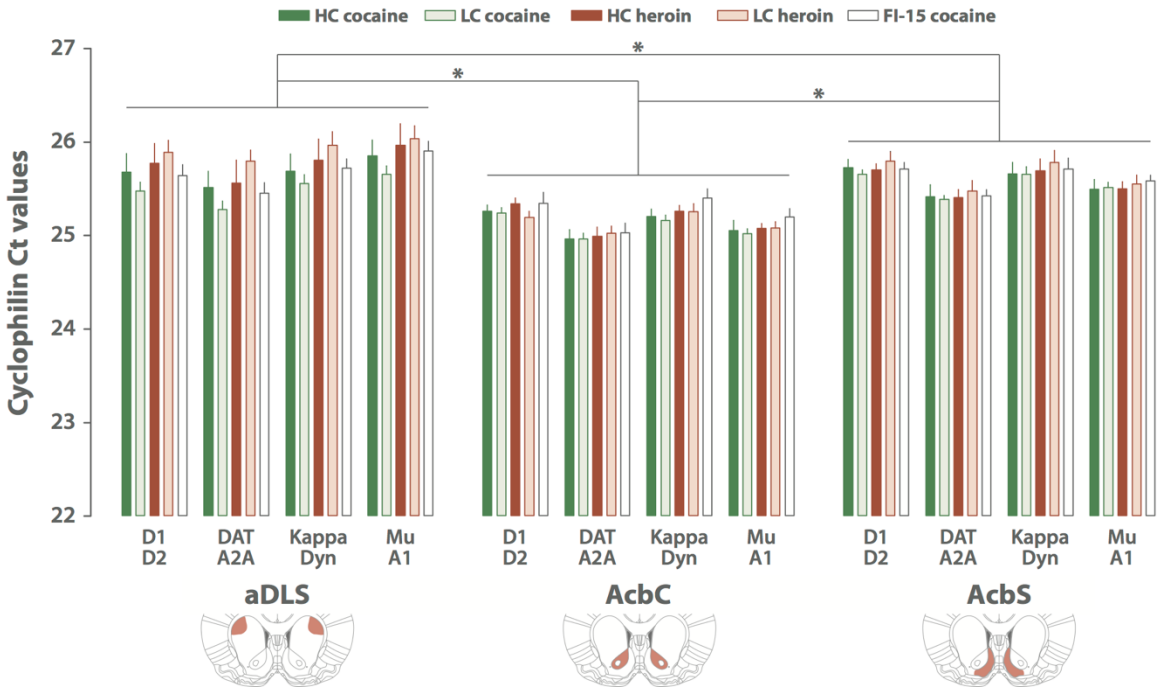


Figure 5.15: Cyclophillin A Ct values calculated for each group within each of the 3 striatal territories. *The Cts for cyclophilin differed between each striatal territory, thereby precluding direct comparison between structures, however, within each structure these Cts did not differ between conditiones and groups. [*: p < 0.05]*

However, as presented in (**Fig. 5.15**) statistically significant differences were observed in the Cyclophillin A Ct between the three striatal structures investigated *[main effect of structure: $F(2,120) = 35.705$, $p < 0.01$]*, precluding comparisons between structures.

However, direct comparison between conditions (SOR vs FI15), drugs (heroin vs cocaine) and groups (HC vs LC rats) were carried out since Cyclophillin A expression was similar across.

Levels of expression of target mRNA in each structure, condition and group are reported as means ± sem in **Table 5.4**. Their analysis revealed specific striatal correlates of compulsive heroin seeking.

a. Striatal substrates of compulsive heroin seeking behaviour

HC rats with an history of SOR for heroin had higher levels of AcbC A2A Rc and D1 Rc mRNA than LC rats *[main effect of group: $F(1,21) = 13.536$, $p < 0.01$* and *$F(1,21) = 6.1825$, $p < 0.05$, respectively]* (**Fig. 5.16**). Since A2A Rcs are able to form heterodimers with D2 Rcs in the striatum, these data suggest that D1 pathway is promoted in the AcbC of HC rats. These differences cannot be attributable to a different pharmacological exposure to heroin since both groups experienced the exact same number of heroin infusions throughout their SA history (**table 5.5**). Interestingly, HC rats also displayed higher levels of dopamien D2 Rc in the AcbC as compared to LC rats, albeit this difference did not reach the threshold for statistical significance (p = 0. 079, **tables 5.4 & 5.5**).

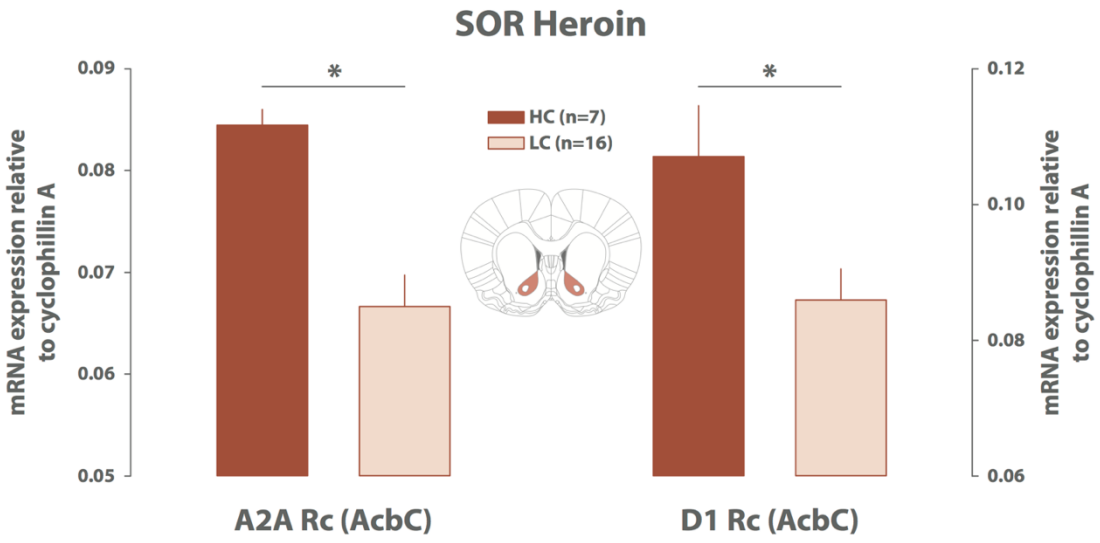


Figure 5.16: Compulsive heroin seeking habits are characterised by a higher leve of A2A Rc and D1 Rc mRNA in the nucleus accumbens core (AcbC). *[*: $p < 0.05$]*

		Overall population			HC : cocaine group			LC : cocaine group			FI-15 : cocaine group			HC : heroin group			LC : heroin group			SOR : cocaine group			SOR : heroin group		
		n	mean	sem	n	mean	sem	n	mean	sem	n	mean	sem	n	mean	sem	n	mean	sem	n	mean	sem	n	mean	sem
DLS	D1 Rc	63	0.1036	0.0032	6	0.1034	0.0079	24	0.1066	0.0050	11	0.1059	0.0100	6	0.1040	0.0116	16	0.0973	0.0056	30	0.1060	0.0042	22	0.0992	0.0051
	D2 Rc	63	0.1340	0.0032	6	0.1287	0.0070	24	0.1343	0.0058	11	0.1300	0.0083	6	0.1379	0.0088	16	0.1368	0.0066	30	0.1332	0.0048	22	0.1371	0.0053
	DAT	63	0.0006	0.0001	6	0.0007	0.0002	24	0.0006	0.0001	11	0.0005	0.0001	6	0.0007	0.0002	16	0.0006	0.0001	30	0.0006	0.0001	22	0.0006	0.0001
	Kappa Rc	61	0.0100	0.0003	6	0.0098	0.0007	22	0.0100	0.0006	11	0.0102	0.0005	6	0.0106	0.0012	16	0.0097	0.0003	28	0.0100	0.0005	22	0.0100	0.0004
	Dyn	63	0.0434	0.0019	6	0.0436	0.0041	24	0.0449	0.0030	11	0.0425	0.0049	6	0.0380	0.0054	16	0.0437	0.0042	30	0.0446	0.0025	22	0.0421	0.0034
	Mu Rc	63	0.0058	0.0003	6	0.0065	0.0012	24	0.0054	0.0006	11	0.0052	0.0005	6	0.0064	0.0014	16	0.0063	0.0007	30	0.0056	0.0005	22	0.0063	0.0006
	A1 Rc	63	0.0173	0.0007	6	0.0166	0.0019	24	0.0184	0.0011	11	0.0171	0.0023	6	0.0156	0.0015	16	0.0169	0.0015	30	0.0180	0.0010	22	0.0165	0.0012
	A2A Rc	63	0.1127	0.0041	6	0.1056	0.0133	24	0.1166	0.0066	11	0.1092	0.0124	6	0.1120	0.0134	16	0.1123	0.0070	30	0.1144	0.0059	22	0.1122	0.0061
AcbC	D1 Rc	64	0.0935	0.0022	6	0.0957	0.0057	24	0.0963	0.0035	11	0.0885	0.0036	7	0.1070	0.0075	16	0.0859	0.0046	30	0.0962	0.0030	23	0.0923	0.0043
	D2 Rc	64	0.0710	0.0016	6	0.0731	0.0049	24	0.0698	0.0023	11	0.0694	0.0035	7	0.0811	0.0070	16	0.0687	0.0033	30	0.0704	0.0020	23	0.0725	0.0033
	DAT	64	0.0013	0.0001	6	0.0016	0.0005	24	0.0015	0.0001	11	0.0013	0.0002	7	0.0012	0.0003	16	0.0008	0.0001	30	0.0015	0.0001	23	0.0009	0.0001
	Kappa Rc	64	0.0159	0.0003	6	0.0156	0.0007	24	0.0159	0.0005	11	0.0149	0.0008	7	0.0176	0.0012	16	0.0158	0.0007	30	0.0158	0.0004	23	0.0164	0.0006
	Dyn	62	0.1461	0.0034	6	0.1399	0.0123	24	0.1557	0.0046	10	0.1455	0.0061	6	0.1415	0.0112	16	0.1362	0.0080	30	0.1526	0.0045	22	0.1377	0.0065
	Mu Rc	64	0.0063	0.0002	6	0.0058	0.0001	24	0.0062	0.0003	11	0.0066	0.0004	7	0.0064	0.0006	16	0.0062	0.0003	30	0.0061	0.0002	23	0.0062	0.0003
	A1 Rc	64	0.0267	0.0006	6	0.0268	0.0017	24	0.0271	0.0008	11	0.0261	0.0009	7	0.0273	0.0017	16	0.0263	0.0015	30	0.0270	0.0007	23	0.0266	0.0011
	A2A Rc	64	0.0667	0.0016	6	0.0684	0.0048	24	0.0637	0.0021	11	0.0612	0.0044	7	0.0845	0.0015	16	0.0666	0.0031	30	0.0646	0.0019	23	0.0720	0.0028
AcbS	D1 Rc	64	0.1070	0.0048	6	0.1012	0.0086	24	0.1055	0.0072	11	0.0918	0.0098	7	0.1413	0.0135	16	0.1069	0.0115	30	0.1047	0.0060	23	0.1174	0.0095
	D2 Rc	64	0.0472	0.0020	6	0.0432	0.0011	24	0.0468	0.0028	11	0.0402	0.0038	7	0.0627	0.0067	16	0.0471	0.0049	30	0.0461	0.0022	23	0.0519	0.0042
	DAT	63	0.0016	0.0001	6	0.0010	0.0003	24	0.0020	0.0002	11	0.0020	0.0002	7	0.0009	0.0002	15	0.0010	0.0001	30	0.0018	0.0002	22	0.0010	0.0001
	Kappa Rc	64	0.0157	0.0006	6	0.0156	0.0017	24	0.0161	0.0011	11	0.0141	0.0010	7	0.0173	0.0019	16	0.0158	0.0012	30	0.0160	0.0009	23	0.0162	0.0010
	Dyn	64	0.1129	0.0061	6	0.0938	0.0053	24	0.1109	0.0099	11	0.0955	0.0099	7	0.1385	0.0128	16	0.1239	0.0165	30	0.1075	0.0081	23	0.1283	0.0120
	Mu Rc	64	0.0076	0.0003	6	0.0082	0.0010	24	0.0078	0.0005	11	0.0076	0.0007	7	0.0072	0.0005	16	0.0075	0.0005	30	0.0079	0.0005	23	0.0074	0.0004
	A1 Rc	64	0.0288	0.0010	6	0.0280	0.0019	24	0.0319	0.0018	11	0.0277	0.0024	7	0.0248	0.0021	16	0.0270	0.0015	30	0.0311	0.0015	23	0.0263	0.0012
	A2A Rc	64	0.0586	0.0023	6	0.0515	0.0045	24	0.0562	0.0031	11	0.0513	0.0045	7	0.0785	0.0071	16	0.0612	0.0057	30	0.0553	0.0026	23	0.0664	0.0047

Table.5.4: descriptive statistics of the Cts of target mRNAs for each condition and group for each striatal structure investigated

		Cocaine : HC Vs LC		Heroin : HC Vs LC		Cocaine : SOR Vs FI-15		SOR : Cocaine Vs Heroin	
		F	*p*	F	*p*	F	*p*	F	*p*
DLS	D1 Rc	0.910	0.765	0.335	0.569	0.001	0.997	1.068	0.306
	D2 Rc	0.205	0.654	0.008	0.929	0.113	0.739	0.300	0.586
	DAT	0.010	0.922	0.232	0.635	0.821	0.371	0.004	0.952
	Kappa Rc	0.024	0.878	1.104	0.306	0.067	0.797	0.001	1.000
	Dyn	0.046	0.832	0.551	0.467	0.182	0.672	0.366	0.548
	Mu Rc	0.743	0.396	0.008	0.928	0.210	0.649	0.724	0.399
	A1 Rc	0.497	0.487	0.232	0.635	0.178	0.676	0.998	0.322
	A2A Rc	0.557	0.462	0.001	0.981	0.184	0.670	0.065	0.799
AcbC	D1 Rc	0.005	0.944	**6.183**	**0.021**	2.010	0.164	0.563	0.457
	D2 Rc	0.421	0.522	3.402	0.079	0.064	0.801	0.294	0.590
	DAT	0.145	0.706	2.433	0.134	0.809	0.374	**10.096**	**0.003**
	Kappa Rc	0.092	0.764	1.827	0.191	1.140	0.292	0.651	0.423
	Dyn	2.067	0.162	0.130	0.722	0.692	0.411	3.830	0.056
	Mu Rc	0.594	0.447	0.068	0.797	1.254	0.270	0.119	0.732
	A1 Rc	0.022	0.883	0.137	0.715	0.525	0.473	0.104	0.748
	A2A Rc	0.997	0.327	**13.536**	**0.001**	0.697	0.409	**5.188**	**0.027**
AcbS	D1 Rc	0.084	0.774	3.050	0.095	1.241	0.272	1.412	0.240
	D2 Rc	0.420	0.522	3.265	0.085	1.856	0.181	1.675	0.201
	DAT	**8.225**	**0.008**	0.033	0.858	0.293	0.592	**19.171**	**0.001**
	Kappa Rc	0.033	0.858	0.471	0.500	1.406	0.243	0.034	0.854
	Dyn	0.713	0.406	0.301	0.589	0.670	0.418	2.233	0.142
	Mu Rc	0.097	0.758	0.157	0.696	0.094	0.761	0.576	0.451
	A1 Rc	1.115	0.300	0.626	0.438	1.394	0.245	**5.661**	**0.021**
	A2A Rc	0.487	0.491	3.096	0.093	0.585	0.449	**4.736**	**0.034**
Total drug infusions		0.051	0.943	0.098	0.757	0.976	0.329	N/A	N/A

Table.5.5: F and p values outcomes of one-way ANOVAs performed on total drug infusions and target mRNA levels in the investigated structures, where conditions (SOR vs FI15), drugs (heroin vs cocaine) and/or groups (HC vs LC) were used as between-subject factors.

b. Striatal substrates of compulsive cocaine seeking behaviour

HC and LC rats with a history of SOR for cocaine did not display the same difference in A2A and dopamine receptors mRNA levels in the AcbC. However, rats showing compulsive cocaine seeking habits had a much lower levels of DAT mRNA in the AcbS than LC rats *[main effect of group: $F(1,28) = 8.2251$, $p < 0.01$]* (**Fig. 5.17**). That difference was indeed very specific to the AcbS as HC and LC rats showed similar levels of DAT mRNA in all the other striatal territories (**Table 5.5**). As previously discussed, this difference cannot be attributable to a differential exposure to cocaine as HC rats did not differ from LC rats in the total number of cocaine infusions received throughout their cocaine self-administration history (**Table 5.5**).

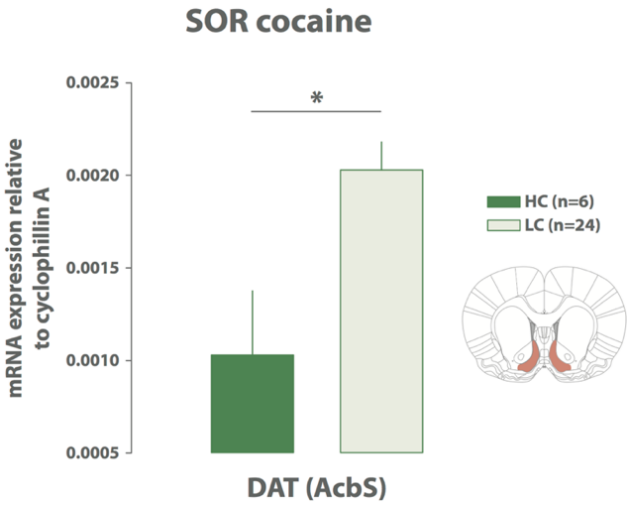


Figure 5.17: Compulsive cocaine seeking behaviour is characterised by a decreased level of mRNA in the AcbS. *Rats with a history of SOR for cocaine that display compulsive behaviour had a much lower level of DAT mRNA in the AcbS than low compulsive rats with the same history of cocaine self-administration. *: p < 0.05]*

Irrespective of the compulsive nature of the drug seeking habit, it appears that incentive habits for heroin vs cocaine were associated with differential adaptations in the striatum. Thus, we further investigated the differences between rats trained under a SOR for heroin and those trained under a SOR for cocaine.

c. *Differences in striatal adaptations between SOR heroin and SOR cocaine*

Incentive habits for heroin were associated with a marked decrease in mRNA levels in the AcbC as compared to incentive habits for cocaine as revealed by the comparison of rats with an history of SOR for heroin, regardless their compulsive phenotypes, to those with a history of SOR for cocaine *[main effect of group: F(1,51) = 10.096, p < 0.01]* (**Fig. 5.18**).

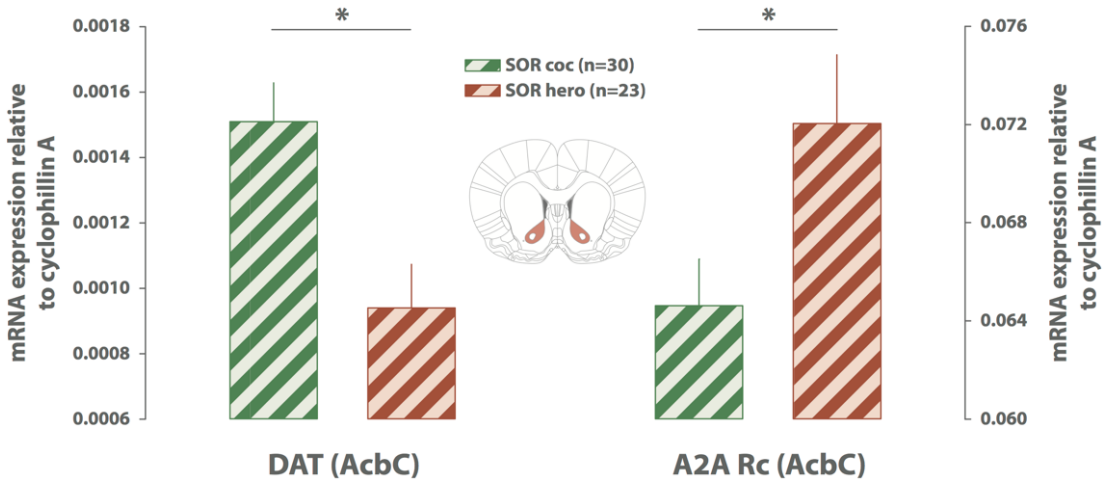


Figure 5.18: DAT and A2A Rc mRNA relative expressions within the AcbC of both SOR cocaine and SOR heroin rats. *DAT mRNA was more expressed and A2A Rc less expressed in SOR cocaine rats compared to SOR heroine rats. [*: p < 0.05]*

In contrast, rats with an history of SOR for heroin displayed a lower level of A2A Rc mRNA in the AcbC than those trained for cocaine under the same schedule [main effect of *group: F(1,51) = 5.1881, p < 0.05]* (**Fig. 5.18**).

These differences observed in the AcbC between incentive habits for heroin and cocaine were paralleled with differences observed in the AcbS in which rats with an history of SOR for heroin also displayed a lower level of DAT mRNA *[main effect of group: F(1,50) = 19.171, p < 0.001]* and higher level of A2A Rc mRNA *[main effect of group: F(1,51) = 4.7360, p < 0.05]* than rats with an history of SOR for cocaine. In contrast, rats with an history of SOR for heroin had lower levels of A1 Rc mRNA than those exposed to the same schedule for cocaine *[main effect of group: F(1,51) = 5.6613, p < 0.05] (***Fig. 5.19**).

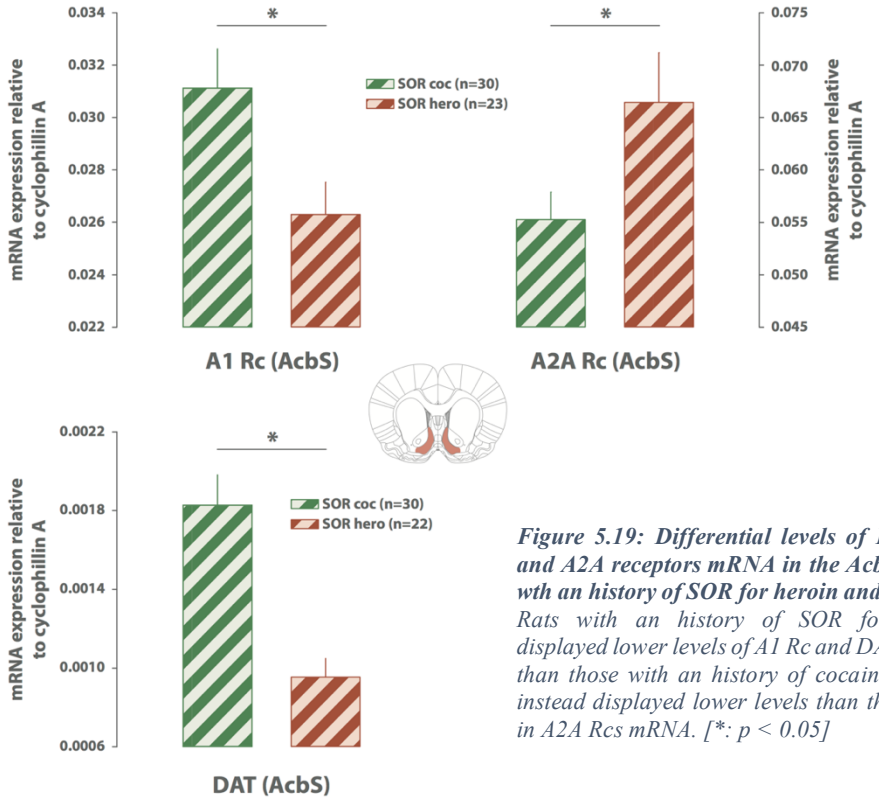


Figure 5.19: Differential levels of DAT, A1 and A2A receptors mRNA in the AcbS of rats wth an history of SOR for heroin and cocaine. *Rats with an history of SOR for heroin displayed lower levels of A1 Rc and DAT mRNA than those with an history of cocaine, which, instead displayed lower levels than the former in A2A Rcs mRNA. [*: p < 0.05]*

Further to these between-subject analyses, we investigated the influence of the amount of each drug rats were exposed to, on striatal adaptations.

4. Correlates between drug intoxication and striatal actors

At the population level, dimensional analyses revealed that some of the levels of expression of these striatal markers were predicted by the amount of exposure to either heroin and cocaine

and dependent on whether rats had developed an incentive habit or not, i.e. whether they had been trained under a SOR or FI15 schedule of reinforcement.

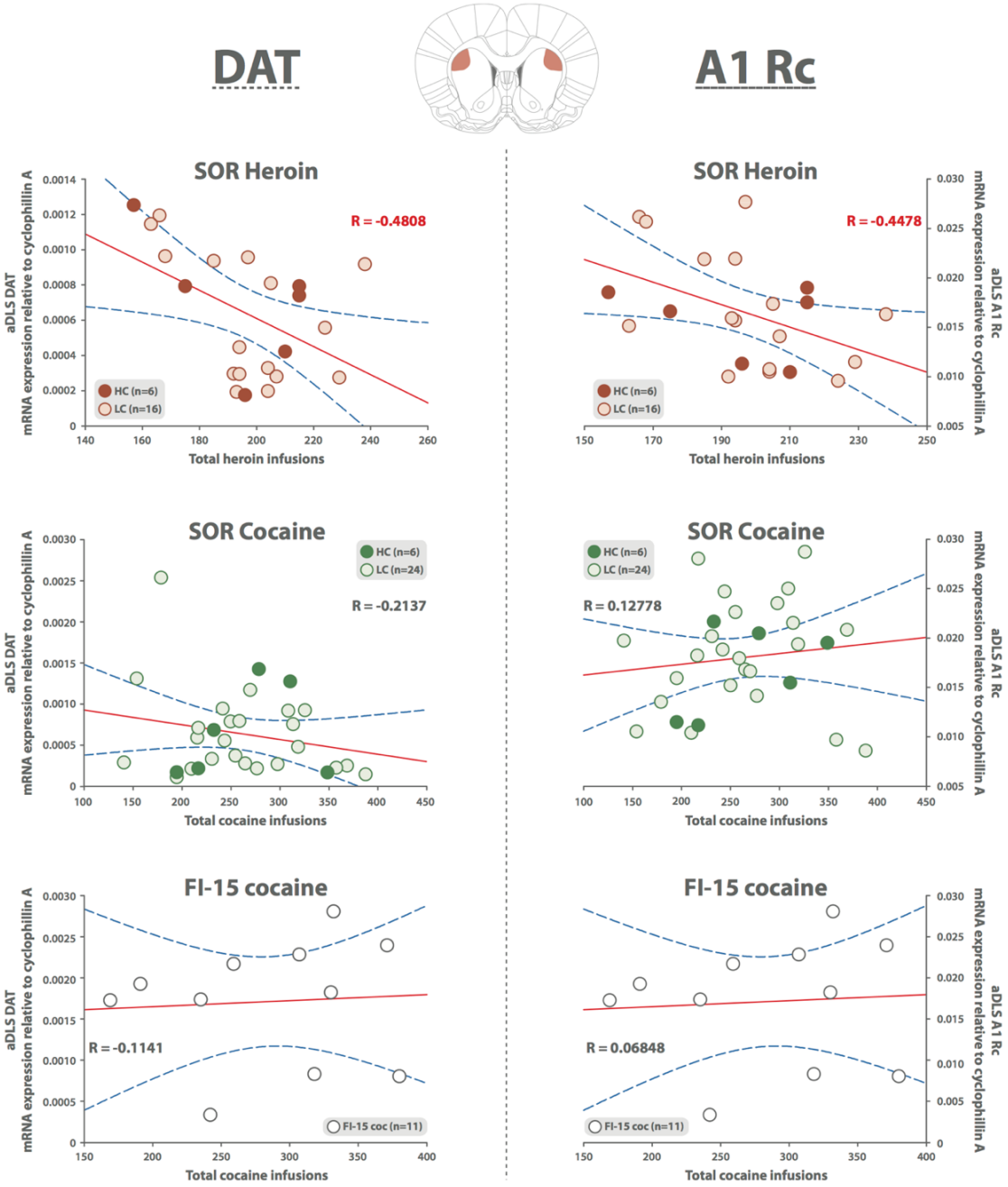


Figure 5.20: Incentive habits for heroin are associated with a decrease in striatal DAT mRNA that is proportional to the number of heroin infusions received throughout the SA history. *Dimensional analyses onpopulations of rats with an history of SOR for heroin, and cocaine and FI15 for cocaine reveal that the level of the DAT and A1 Rc mRNA in the aDLS following heroin SA is predicted by the number of heroin infusions throughout the SA history only in rats with a SOR training.*

Thus, adaptations in DAT and A1 Rc mRNA levels in the aDLS were predicted by the number of heroin infusions received throughout the SA history exclusively in rats that had been trained

under SOR for heroin (**Fig. 5.20**), suggesting that an interaction is necessary between the cellular mechanisms involved in the direct pharmacological effects of heroin and those underlying the development of incentive habits for these biological adaptations to be recruited in the aDLS.

Together with the evidence that the decrease in the levels of dynorphin and D1 Rc mRNA in the AcbS were predicted (negative correlation) by the total number of infusions received by rats trained under FI15, but not SOR for cocaine (**Fig. 5.21**), these data offer evidence that these striatal adaptations are predicted by the overall exposure to the drug, but gated, or enabled, by the history of training, differentially leading to the emergence of drug seeking habits or incentive habits.

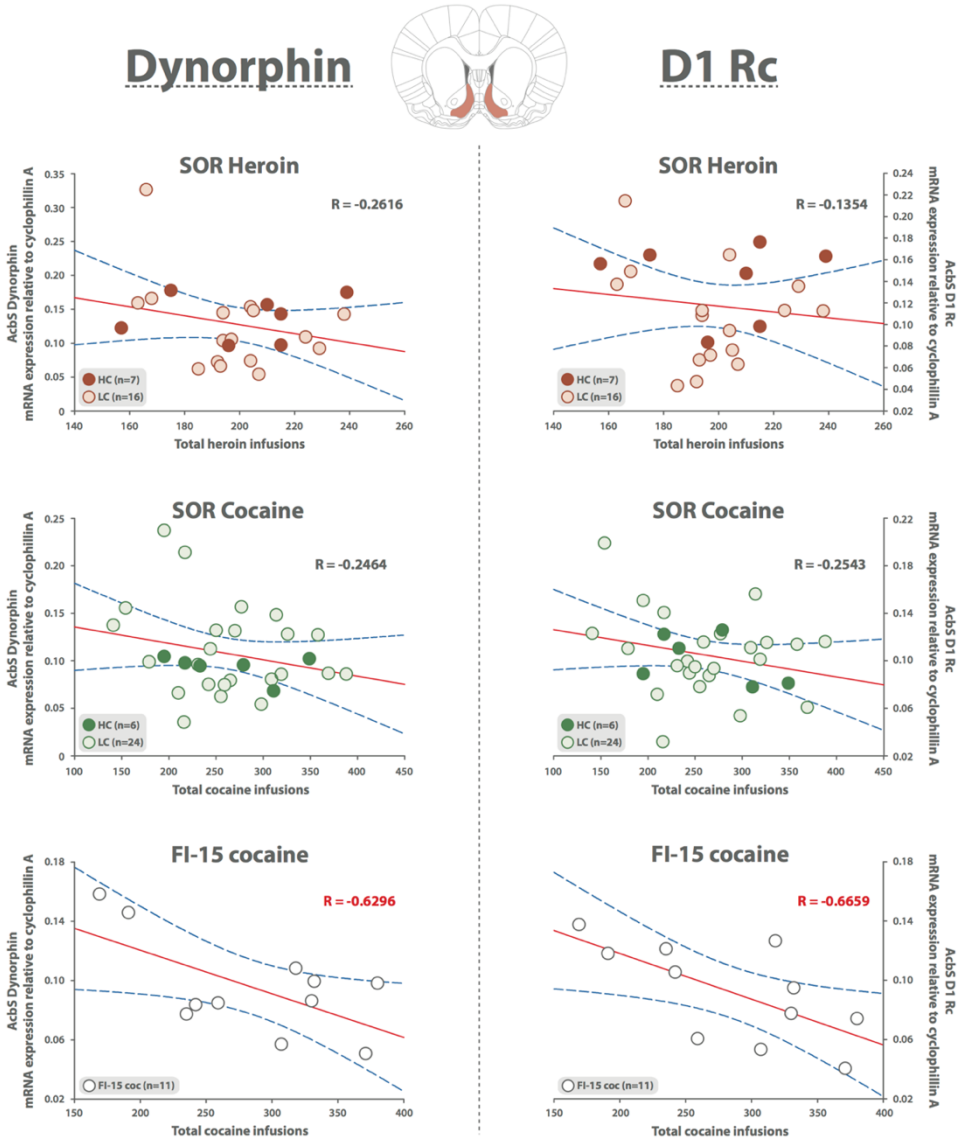


Figure 5.21: Dynorphin and D1 Rc mRNA levels in the AcbS negatively correlate with the total drug infusions received, but exclusively in rats with an hisotyr of training under a FI-15 schedule of reinforcement for cocaine.

Thereby these data further support the importance of the psychological mechanisms involved in drug seeking in shaping the neurobiological adaptations to the exposure to the same amount of drug, as observed between SOR and FI15 for cocaine.

The decrease in dynorphin mRNA as a function of past cocaine exposure was also present within the AcbC of rats with an history of training under a FI15 schedule of reinforcement (**Fig. 5.22**).

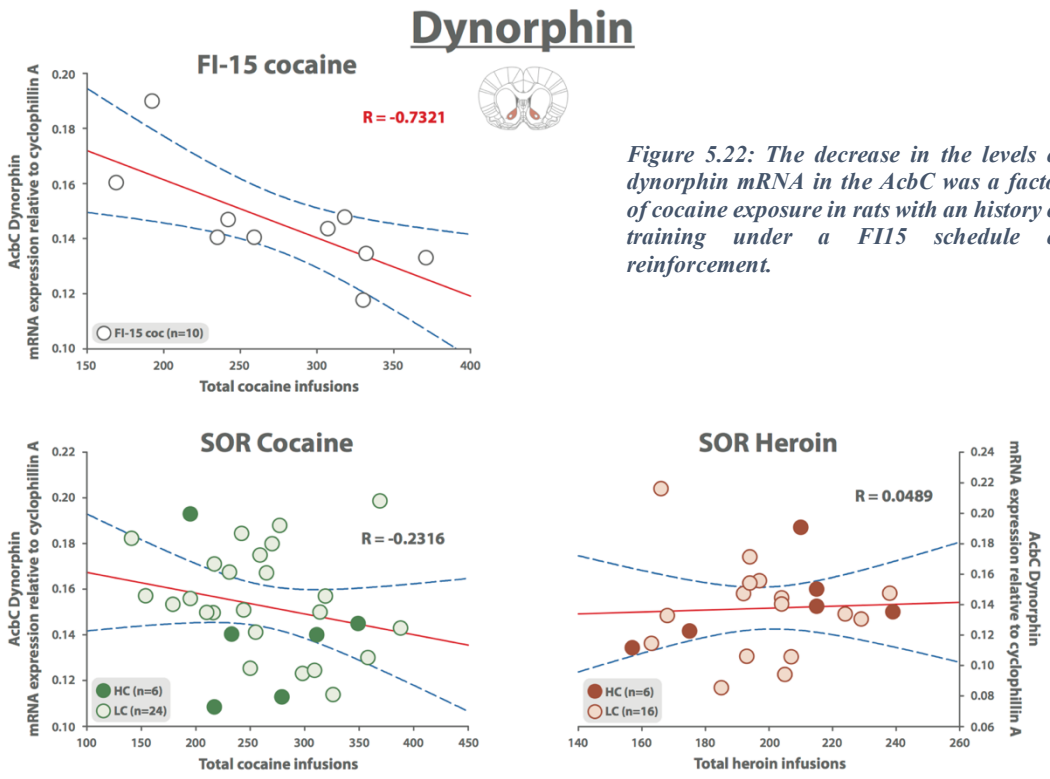


Figure 5.22: The decrease in the levels of dynorphin mRNA in the AcbC was a factor of cocaine exposure in rats with an history of training under a FI15 schedule of reinforcement.

Discussion

The series of experiments presented in this chapter were carried out in order to address two main questions: *1)* is there a "functional signature at the neural level" of incentive habits for heroin and associated vulnerability to compulsivity and *2)* Can we identify potential cellular correlates in the striatum among candidate markers involved in the physiology of the striatal dopaminergic synapse.

The hotspot analysis carried out using in situ hybridisation targeting zif268 mRNA has been a successful strategy to investigate the differential recruitment of functional neural networks in rats expressing drug seeking as a habit, an incentive habit or a compulsive incentive habit. Indeed,

the differential patterns of expression of zif268 observed in the results of this chapter were exclusively dependent on the behavioural challenge the rats had been exposed to, 45 min prior to sacrifice (namely a 15 min drug seeking challenge in a drug-free state). These 45 min offered time to cellular plasticity-related mechanisms to occur, reflected by zif268 mRNA levels [353, 409].

The differences observed between rats with a history of SOR and those with a history of FI15 de not appear attributable to a differential level of instrumental responding at test. No correlations were observed between performance at test (drug seeking responses, as measured as active lever presses during the 15 min drug free period) and the levels of the zif268 mRNA within the corticostriatal circuitry. However, correlations were observed in specific structures between the total number of drug infusions received throughout the SA history and zif268 mRNA levels at test, which can only be explained by an influence of the interaction between the intoxication history of the individual and the recruitment of a specific psychological mechanism, and associated neurobiological substrate, in the performance of drug seeking at test. Such conclusion leaves little room for a potential confound by differences in response rates at test. Nevertheless, one potential way to parametrically control for this would be to match rats from both conditions (SOR vs FI15) on their level of responding and ensure that the difference in the pattern of expression in zif268 as observed here survives this direct comparison. Clearly many more individuals would be needed to perform such a control, which, I believe is not necessary, for the reasons mentioned above and the specificity of the differences observed as discussed below.

The analysis of the levels of zif268 mRNA by in situ hybridisation throughout the entire corticostriatal circuitry of rats exposed to a SOR or FI15 schedule of reinforcement for heroin offered unprecedented insights into the neural substrates of incentive habits and compulsive heroin seeking behaviour. Thus, when compared to heroin seeking habits, incentive habits were characterised by higher recruitment of cellular plasticity in a network involving the IL cortex and the aDLS. These structures have both been shown to be necessary for the control over instrumental responding by S-R associations [410, 411] and the latter to be the necessary neural output of an amygdalo-cortico-striatal circuitry mediating the development and expression of

incentive habits for cocaine [146, 245] and heroin (unpublished data) [336]. These data support the hypothesis that incentive habits are indeed an aberrant form of habit, the expression of which may engage plasticity mechanisms in the IL-aDLS network to process the inputs from the amygdala that mediates the conditioned reinforcing properties of the CS.

This hypothesis is supported by the specific correlation in the level of zif268 mRNA observed in rats displaying incentive habits for heroin, between the BLA and two key amygdalo-striatal partners involved in incentive habits, namely the CeA and the AcbC. The functional convergence in cellular plasticity engaged during a drug seeking episode observed between the BLA and the CeA is reminiscent of the evidence that a functional transition occurs within the amygdala, from the BLA to the CeA, between the development and long-term maintenance of incentive habits for cocaine [245]. Whether the BLA is necessary for the long-term expression of incentive habits for heroin remains to be tested. However, the present data suggest that it undergoes plasticity during the performance of these incentive habits, and that the magnitude of this plasticity is linearly related to the one observed in the CeA, to which it projects, and the AcbC, through which it gains access over the function of the aDLS eventually to recruit its control over drug seeking [245]. Thus, incentive habits for heroin are shown to recruit cellular plasticity in a neural network that involves the BLA, the AcbC, the CeA and the aDLS, which is precisely the circuit identified, using a series of causal manipulations, including functional disconnections, for incentive habits for cocaine [146, 245]. Consequently, the core neural basis of incentive habits may be similar between the two drugs.

However, the expression of an incentive habit for heroin was also associated with convergent plasticity mechanisms between the BLA and the PL cortex, and between the BLA and the AcbS, the latter being, alongside the aDLS, one of the few regions in which cellular plasticity is more recruited by the expression of an incentive habit for heroin. In these incentive habits, increased cellular plasticity is observed in the IL cortex, and not the PL cortex (at least at the statistical threshold taken for significance here). These data indicate that the IL cortex is actually more recruited in incentive habits, potentially reflecting its control over, but not involvement, in a network involving the PL cortex (which is involved more in flexible, goal-directed behaviour than habits) and the BLA which functionally converges during a heroin seeking episode.

Together with the correlation in cellular plasticity observed between the BLA and the AcbS, the latter showed no correlation with the other striatal territories, therefore, this observation suggests that incentive habits may reflect the convergence of two streams of information, originating in the BLA. A reflexive, sensory specific stream, through the AcbS and the CeA, potentially under direct control of the IL cortex, and one more motivational, cognitive stream, through the AcbC which is under the influence of the PL cortex [412]. Both would eventually bring the cognitive motivational and sensory-specific properties of the CS to the aDLS to aberrantly engage a S-R mechanism, the response component of which being imbued with the motivational value of the CS. The lack of differential activation of zif268 in the PL and AcbC of rats expressing an incentive habit suggests that the AcbC and associated PL are not necessarily undergoing any form of processing in a rat engaged in heroin seeking when the behaviour is governed by incentive habits. This is in line with the suggestion that the critic function of the AcbC [413, 414] is disrupted, such that it merely channels the information it receives from the amygdala to the aDLS, supposedly via the spiralling ascending dopamine circuitry [146, 415]. This interpretation may also help reconcile the lack of correlation in zif268 levels observed here between the ventral and dorsal striatal territories and the evidence that the functional coupling between the ventral and dorsal striatum seems increased in former heroin addicts [416].

Alongside the lack of differential recruitment of the aDMS and the several territories of the OFC, these data suggest that incentive habits for heroin do not stem from a deficient goal-directed system as compared to heroin seeking habits (as it has been suggested for other conditions [417]), but instead from an aberrant engagement of subcortical Pavlovian and S-R instrumental mechanisms [145].

Importantly, no correlations were found between ZIF-268 mRNA levels in all the investigated structures and previous behavioural performance, such as total active lever responses and number of CSs presentations displayed the during the drug-free seeking session preceding the sacrifice, suggesting that the adaptations discussed above are quantitatively distinct from the rate of responding or CSs presentations.

Yet, both in the BLA and CeA, the mRNA levels of zif268, as measured after a drug-free heroin seeking challenge, were predicted by the total amount of heroin each individual had self-

administered with an incentive habit, but not a history of seeking under a FI15 schedule of reinforcement. When compared to the observation that the level of zif268 at test in the PL cortex was predicted by previous drug history irrespective of the training conditions, these data offer insight into the long-term consequences of drug exposure onto subsequent functional recruitment of prefrontal structures. A rather non-specific effect that is in contrast with the amygdala, wherein, both in the BLA and CeA, the cellular plasticity taking place at test was predicted by previous drug history, but only in rats with an incentive habit. This suggests that the direct pharmacological effects of the heroin, which should be impervious to the nature of the psychological process that governs drug seeking, namely incentive habit vs habit, only permeated that amygdala territories in conditions under which it was actively engaged during drug seeking. Thus, the amount of heroin self-administered only influenced subsequent cellular plasticity at test in the amygdala of rats whose previous drug seeking behaviour was mediated by the functional engagement of these brain regions. This is, I believe, one of the most striking results of this work as it suggests that, for heroin to neurobiologically influence the subsequent functional recruitment of brain structures upon drug seeking, these structures must have been functionally involved when heroin is being self-administered.

Interestingly, this hotspot analysis did not offer any specific neural signature of compulsive heroin seeking behaviour. Indeed, HC rats differed from LC in the levels of Zif28 in none of the structures investigated here, including the aDLS and insula, which have both been shown to be important in the expression of compulsive behaviours [332, 348]. This may not be surprising since the compulsive nature of the behaviour was not necessarily triggered behaviourally under these challenge conditions. Thus, future studies are required to better investigate the differential recruitment of these structures in rats seeking the drug under the threat of punishment (as observed during the first half of the interval of punished sessions) or just resuming their drug seeking behaviour after cessation of punishment.

Nevertheless, compulsive heroin seeking behaviour was associated with specific adaptations in the expression of candidate genes within various territories of the striatum.

HC rats with a history of training under a SOR for heroin (SOR-heroin-HC rats) had a much higher level of expression of the A2A and D1 Rc in the AcbC as compared to LC rats with the same

history. Even if these differences were not reflected in the differential activation of zif268, they suggest that the balance between D1 and D2 signalling in the AcbC may be biased. As previously mentioned, A2A Rc form heterodimers with D2 Rc on MSNs, thereby decreasing the affinity of dopamine for the latter. Combined with the increased D1 mRNA levels, which may reflect an increased availability of the protein at the membrane (this will need to be confirmed in future experiments), the integration of dopamine signalling in the AcbC may be biased towards the facilitation of behaviour associated with D1 receptors which also contribute to the rewarding properties of drug of abuse [418-420].

Since adenosinergic signalling involves not only neurons, but also astrocytes, these observations may reflect adaptations that take place beyond the classical view of neuronal ensembles, reflecting a dynamic contribution of astrocytes to the integration of striatal dopamine signalling. Further research is warranted to better understand the contribution to compulsive heroin seeking behaviour of the interaction between the adenosinergic and dopaminergic mechanisms in the AcbC. One particular issue, which also applies for the following observations, is whether these inter-individual differences predate the exposure to heroin or whether they result from the interaction of a specific vulnerable phenotype with heroin in rats that have developed an incentive habit for heroin.

However, compulsive heroin seeking behaviour was not found to be associated with lower levels of the D2 mRNA in any of the striatal territories. This seems to be at odds with the wealth of literature that has reported a decrease in striatal level of D2/3 dopamine receptors, as measured using PET imaging in humans and primates [135, 137] or in situ hybridisation in rats with an extended history of cocaine self-administration [330]. It may well be the case that all rats in this study would display lower D2 dopamine receptor levels in the striatum as compared to drug-naïve rats. Future studies will be necessary to address that hypothesis. Nevertheless, the present observation does suggest that the emergence of compulsive heroin seeking is not causally related to an alteration in the transcriptomic or post-translational regulation of this receptor. It may also illustrate a lack of functional relationship between mRNA and protein levels, even if the lower level of dopamine D2/3 receptor binding identified in the ventral striatum of high impulsive rats, which are highly vulnerable to develop compulsive behaviours, was shown

to be dependent both upon pre (in VTA dopamine neurons) and post-synaptic (in MSNs) decrease in the mRNA levels [330] .

As compared with compulsive heroin seeking behaviour, compulsive cocaine seeking behaviour, in rats with a history of training under a SOR for cocaine, was characterised by a marked decrease in the DAT mRNA levels in the AcbS. This is another striking result of this investigation as it reveals that the DAT expressed on astrocytes within the ventral striatum is indeed associated with the vulnerability to compulsively to seek cocaine. Indeed, the mRNA of the DAT expressed by dopamine neurons is located in their cell body, in the mesencephalon. It is very abundant and the protein in these neurons contributes to regulate the somatodentritic concentration of dopamine as well as the synaptic concentration of dopamine in the target regions [421]. The mRNA detected here in the striatum was expressed at very low levels, relative to the other markers investigated in this study. If in mammalian adult brains, some mRNAs have been sporadically detected in axoplasm [422, 423], they are mainly involved in the maintenance and repair of neuronal assembly [422]. Therefore, the DAT mRNA detected in the striatum in this study is very likely expressed in astrocytes (see chapter 6 for further discussion). A decrease in dopamine reuptake by astrocytes in the AcbS would result in a loss of regulation of the spread of the dopamine transmission (volume transmission) in the ventral part of the striatum, whereby extracellular levels of dopamine brought about by drug exposure would recruit and functionally control entire striatal territories. Thereby this would facilitate the recruitment of adjacent, dorsal, striatal territories and a dopamine-dependent functional coupling of the ventral and dorsolateral striatum. This observation has led me to further investigate this particular contribution of DAT adaptations in astrocytes in an independent set of studies that are presented in chapter 6.

That difference in striatal DAT mRNA levels was only observed between HC and LC rats only for cocaine, and there were several striatal adaptations that were selective to a particular drug, highlighting the differences that exist between stimulant and opiates in the neurobiological adaptations they trigger [339]. Thus, while compulsive cocaine seeking behaviour was characterised by a lower level of DAT in the AcbS, it appeared that SOR-heroin rats overall, had a lower level of DAT mRNA in the AcbS and AcbC than SOR-cocaine rats. This observation suggests that the decrease in DAT mRNA levels in the striatum is not just a cellular adaptation to the direct

interaction of cocaine with the protein, which could have triggered a cascade of downstream events leading to a decrease in its expression. Instead, it suggests that a decrease in DAT mRNA levels occurs in response to hyperdopaminergic states, which may be of higher magnitude after heroin than cocaine at the doses tested. This requires further investigations but opens avenues for understanding the mechanisms of vulnerability to compulsive cocaine seeking.

Similarly, the A2A Rc levels were higher in the AcbS and AcbC of SOR-heroin rats than SOR-cocaine rats, suggesting a potential preferential functional recruitment of the D1 pathway in the former. This hypothesis is further supported by the observation that SOR-heroin rats also display lower levels of A1 Rc mRNA in the AcbS. Since A1 Rcs form heterodimers with D1 receptors and decrease the affinity of dopamine for the latter, these adaptations in the AcbS of hero-SOR rats converge towards an imbalance in the D1 and D2 signalling in the ventral striatum towards the former.

Interestingly, these adaptations in the mRNA level of the D1 dopamine receptor and potential associated functional consequences, as discussed below, were not associated with similar quantitative adaptations in the mRNA level of the peptide expressed by D1-containing neurons, namely dynorphin. Dynorphin, the expression of which has been shown to be regulated by the activity of D1 dopamine receptors, greatly influences dopamine release and post-synaptic integration of dopamine signalling [424]. Its recruitment by addictive drugs and the associated activation of its receptor, the κ-OR, has been associated with the dysphoric properties of withdrawal and the escalation of cocaine self-administration [191, 425]. Importantly, prodynorphin mRNA levels have been shown to increase in both the ventral and dorsal territories of the striatum exposure to drugs, including several weeks of heroin or cocaine SA under fixed ratio schedule of reinforcement [379, 426, 427]. Yet, in primates trained to self-administer cocaine under Fixed interval 3 min schedule these differences in dynorphin levels were not observed in the ventral striatum [428], thereby suggesting that the drug-induced alteration in dynorphin levels are very much dependent on the schedule of reinforcement.

In the present study, a decrease in dynorphin mRNA levels was shown to be linearly related to the total number of cocaine infusions rats had received throughout their history of SA, but exclusively if that history was under a FI15 schedule of reinforcement. This drug-specific, but also

schedule-specific adaptation, suggests that the conditioned reinforcing properties of the CS presented contingent upon responding, or the associated neural mechanisms engaged in the establishment of incentive habits for cocaine, cancel-out the adaptations observed in the rats with a history of training under FI15. As previously described, dynorphin is synthesised by D1-expressing MSNs and potentially astrocytes and binds to κ-ORs. The activation of κ-ORs located on dopaminergic terminals inhibits dopamine release. Thus, the drug-related decrease in dynorphin observed in FI15 rats may reflect an adaptation to vigorous responding for long periods of time with no reinforcement, whereby dopamine release may be facilitated to sustain the effort of doing so [429-431]. However, I would speculate that the absence of such adaptation in the SOR cocaine group may reflect the ability of the CSs to bridge delays to reinforcement, acting as reinforcers, and thereby decreasing the associated effort-related facilitation dopaminergic transmission. This clearly warrants future research.

Surprisingly, no differential adaptations in the mRNA levels of the candidate genes were observed between compulsive and non-compulsive rats in the aDLS, the neural locus of control over habits and the final output of incentive habits. Yet, a decrease in the mRNA levels of the DAT and A1 Rc within the aDLS was shown to be predicted by the number of infusions rats with an history of training under a SOR for heroin, but not for cocaine (irrespective of the self-administration schedule of reinforcement). This suggests that the aDLS is not immune to alterations in the key markers of the regulation of dopaminergic transmission, but that we are yet to identify those that are selectively associated with the vulnerability to develop compulsive heroin seeking habits.

Further investigations of the transcriptomic profile of the structures of the corticostriatal circuitry, including the prefrontal cortex, the amygdala, the pDMS and the insular cortex to name a few, are warranted better to understand the nature of the cellular basis of incentive habits and compulsive heroin seeking. Additionally, direct comparison with drug naïve animals would be an optimal strategy further to probe whether the differential expression profile of the candidate genes between the conditions and subgroups reflect down- or upregulations as compared to baseline and whether these differences also predate the exposure to heroin.

Nevertheless, this series of experiments offered unprecedented insights into the neural and

cellular basis of compulsive heroin seeking behaviour, especially identifying the potential involvement of striatal astrocytes in the dopamine-associated alterations that are observed in drug addiction.

CHAPTER 6: CONTRIBUTION OF ASTROCYTES TO INTRASTRIATAL FUNCTIONAL SHIFTS OVER THE COURSE OF DRUG EXPOSURE?

Introduction

As extensively described in the previous chapters, the neurobiological mechanisms supporting the protracted nature of incentive habits and associated intrastriatal functional shifts remain to be established. Whereas most of the adaptations observed within the striatum in response to drugs, particularly cocaine, exposure have been suggested to stem from neurons, they might be dependent upon astrocytes which have been increasingly suggested to be implicated in psychiatric disorders including drug addiction.

Astrocytes express a broad range of neurotransmitter receptors on their membranes including dopaminergic [432], endocannabinoids [433], GABA [434], glutamate [435, 436] and noradrenaline [437, 438] receptors. The activation of these receptors triggers direct modulation of intracellular calcium levels [for a detailed review see 439]. Changes in calcium levels can be restricted locally or can, when large enough, affect more distant astrocytes through calcium waves [440, 441] that propagate through inter-astrocytic gap junctions that are mostly constituted of connexins 43 and 30 [442]. Modulation of intracellular calcium levels in astrocytes can eventually trigger the release of neurotrophic factors, known as gliotransmitters, such as ATP, D-serine, TNF-alpha or even glutamate, which all play an important role in synaptic activity [443-445].

Importantly, not only do astrocytes release these gliotransmitters, thereby influencing neighbour neurons by which they are also regulated, but they play a key regulatory role in glutamate homeostasis. Thus, beyond releasing glutamate upon activation by calcium waves, astrocytes ensure extracellular levels of glutamate are tightly controlled. Following neuronal release, glutamate is cleared from the synaptic cleft via astrocytic transporters (namely GLT-1 and GLAST) [446]. Glutamate is then converted in glutamine in the intracellular compartment by the enzyme glutamine synthetase [447] and released in the extracellular space, making it

available for neurons as a precursor of the synthesis of glutamate (**Fig. 6.1**).

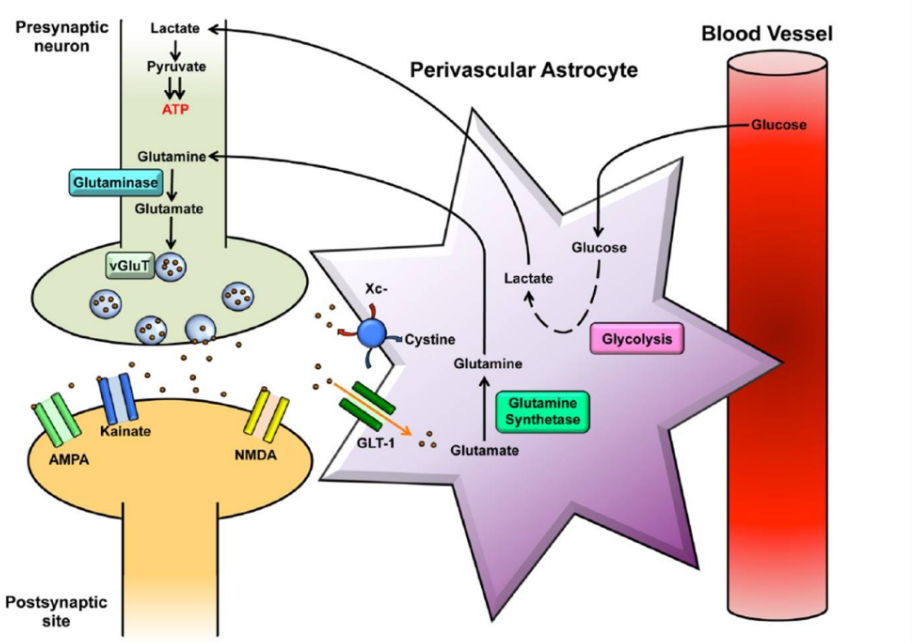


Figure 6.1: The tripartite glutamatergic synapse. *Following an action potential, glutamate is released in the synaptic cleft where it triggers post-synaptic responses via its interaction with ionotropic glutamate receptors. The termination of glutamatergic signalling is mediated by its uptake carried-out by GLT-1 expressed on astrocytes. Then, glutamate is either released in the synaptic cleft via an exchanger (xCT) or converted into glutamine in the astrocytic cytosol. Glutamine is then made available as a precursor for neurons to sunthetise glutamate. Astrocytes also provide energetic supplies to neurones by releasing lactate (a by-product of glucose metabolism) in the extracellular space. From [5]*

Not surprisingly, considering their key role in regulating glutamate homeostasis and the function of the glutamatergic tripartite synapse, astrocytes have been shown to be associated with, or involved in, the pathophysiology of several neuropsychiatric conditions such as bipolar depression [448-452], schizophrenia [453, 454] and drug addiction [5, 455-459].

In the context of addiction, astrocytes have been suggested to play an important regulatory function in the synaptic junctions within the AcbC and the DLS. Consequently, astrocytes potentially contribute to the alterations of glutamate homeostasis within the corticostriatal circuitry that are associated with exposure to, and withdrawal from, cocaine [182]. This is far reaching when one considers the large anatomical and functional territories one astrocyte is able to regulate, since a single astrocyte in the rat can contact up to 140000 synapses [460]. This would suggest, in the cytoarchitectonic context of the striatum, that a single astrocyte could define a functional territory, or facilitate the functional recruitment of adjacent territories, defined on anatomy and connectivity.

Treatment with N-acetylcysteine (NAC), the cysteine prodrug which acts as a substrate for the cysteine/glutamate antiporter xCT, decreases habitual control over behaviour [461] as well as aDLS-dependent incentive habits for cocaine and heroin [462]. NAC which shows promising therapeutic properties in humans, has been shown, at the cellular level, to rescue cocaine-induced-decrease in GLT-1 protein levels both in the AcbC [463], which has been considered until very recently to be its primary site of action, and in the aDLS [315].

Since GLT-1, the molecular target of NAC is only expressed onto astrocyte membranes [464, 465], these observations suggest that astrocytes, potentially through the regulation of the striatal glutamate-dopamine synapse, are involved in the regulation of the functional recruitment of the aDLS-dependent mechanisms, discussed previously to be involved in addiction. In assessing the potential contribution of astrocytes to the cellular and molecular correlates of incentive habits and compulsive heroin seeking habits identified in the previous chapter, it is important to highlight that astrocytes also control extracellular levels of glutamate by its direct release triggered by stimulation of adenosine A2A receptors (A2A Rcs) [466, 467, for a detailed review see 468]. Astrocytes also influence glutamate homeostasis via ð-ORs, the activation of which results in the up-regulation of excitatory amino acid transporters in astrocytes [469], thereby representing a potential converging mechanism between endogenous opiates and astrocyte-mediated regulation of synaptic plasticity within the striatum. Finally, CB1 receptors, which are densely expressed in the striatum, are also present at the surface of the membrane of astrocytes and their activation via neuronal endocannabinoids generates astrocytic glutamate release, phenomenon that has been recently shown to be involved in the homotypic potentiation of medium spiny neurons in the striatum [470].

While the pivotal role of astrocytes in glutamate homeostasis has been described fairly well, the contribution of these cells to dopamine homeostasis remains poorly understood despite increasing evidence from both *in vitro* and *in vivo* studies supporting a role for astrocytes in the dopaminergic synapse. Thus, a better understanding of the role of astrocytes in the regulation, not only of the prototypic tripartite glutamatergic synapse, but also of the striatal dopaminergic synapse is paramount for our understanding the striatal cellular processes involved in addiction.

Under physiological conditions, that are differentially hijacked by cocaine and heroin as

discussed in the general introduction, following an action potential or the activation of presynaptic receptors, a sudden increase in calcium concentration in the presynaptic dopamine terminal triggers the release of dopamine into the synaptic cleft. There, dopamine binds to its post-synaptic receptors, directly facing the terminal in the synaptic cleft in the case of D2 dopamine receptors, or slightly outside it in the case of D1 dopamine receptors as well as pre-synaptic dopamine D2 receptors. The termination of dopaminergic signalling is governed par two mechanisms: a specific recapture of dopamine via the DAT [27] expressed at the membrane of both neurones and astrocytes [28, 331], and enzymatic catabolism of dopamine by the monoamine oxydases (MAO) and the cathecol-o-methyltransferase (COMT) that are not only expressed in neurones and astrocytes [29, 30] but also, at least for the COMT, located within the synaptic cleft (**Fig. 6.2**).

Apart from their role in dopamine clearance, cultured astrocytes have been shown to respond to dopamine in that it influences cytosolic calcium signalling. The influence of dopamine over the function of astrocytes has hitherto been shown to be mediated by two distinct mechanisms. Firstly, through direct activation of dopaminergic D1/D2 receptors that modulate astrocytic calcium signalling [471]. Secondly, as shown by Vaarmann and colleagues, in a receptor-independent manner, whereby the reactive oxygen species generated by the cytosolic degradation of dopamine by MAO directly control calcium signalling [472], suggesting an important role of DAT, the gateway for dopamine to enter the astrocytes, in this process.

The exclusive contribution of astrocytic DAT activity to dopamine homeostasis remains largely undescribed. However, its peri-synaptic location, similar to the one of D1-like dopamine receptors [473, 474], supports the hypothesis that astrocytic DAT contributes to the local regulation of dopamine volume transmission, restricting the spread of influence of each single synapse outside its cleft. This under investigated mechanism may play a very important functional role in the context of dopamine spill over as it is the case following administration of addictive drugs.

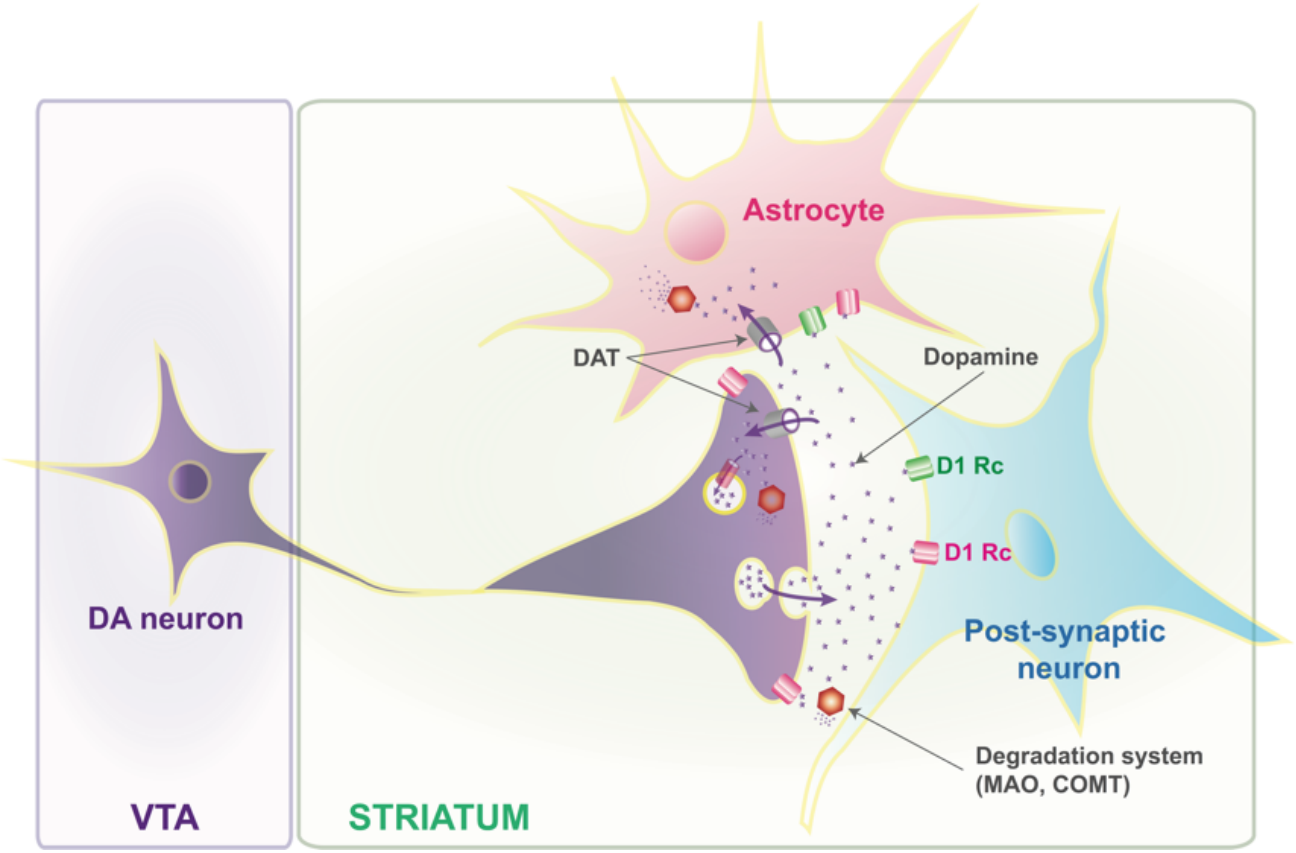


Figure 6.2: The tripartite dopaminergic synapse. *Following an action potential, dopamine is released in the synaptic cleft where it triggers post-synaptic responses via its interaction with dopaminergic D1- or D2-like receptors. The termination of dopaminergic signalling is mediated by its uptake by the dopamine transporter (DAT) expressed both on dopamine neurons and astrocytes, or the slower process of degradation by enzymatic reaction (mediated by COMT or MAO).*

The progressive recruitment of the dorsolateral striatum over the course of drug exposure as previously described has also been shown at the level of the density of the DAT in non-human primates [328] and the data presented in the previous chapter seem to reveal a potential important contribution of alterations in astrocytic DAT in the processes subserving both habitual and compulsive drug seeking behaviour.

In this context, and as previously mentioned, if astrocytes are both involved in local regulation of the spread of dopamine volume transmission and interact with hundreds or thousands of synapses within the striatum, they may offer a unique cellular mechanism whereby adjacent territories of the striatum can become functionally coupled, or facilitate the functional recruitment of the aDLS dopamine-dependent mechanisms.

It was therefore speculated that alterations in the expression of the DAT on striatal astrocytes

may predate the functional recruitment of aDLS control over behaviour in rats instrumentally responding to seek addictive drugs, but not natural reinforcers.

Thus, in this study, rats were trained to self-administer either cocaine or heroin under a SOR schedule of reinforcement. Animal's brains were processed to assess DAT protein levels by western blot from micro punches of striatal territories (containing both neurons and astrocytes) or from primary astrocytes cultured from the same territories, to investigate the contribution of astrocytes to the potential modulation of striatal DAT density induced by (or contributing to) the development of incentive habits.

In order to test if any adaptations observed occurred prior to the onset of incentive habits, or, at the neural systems level, prior to the functional recruitment of aDLS control over behaviour, and were indeed specific to addictive drugs, other rats were trained to self-administer cocaine, heroin or food pellets under a FR-1 schedule of reinforcement long enough for them to have self-administered as many reinforcers as those with a history of training under SOR. While these conditions maintain pDMS control over behaviour [202] they enable to control for a potential differential exposure to the drug.

Materials and methods

1. Behavioural training

In order to investigate the involvement of DAT in the intrastriatal functional shifts that subserved the development of maladaptive drug seeking habits, rats (n=50) were subjected to drug, i.e. cocaine (n=23; 0.25mg/infusion) or heroin (n=20; 0.04mg/infusion), self-administration under a SOR in the operant boxes described in chapter 2. Rats were initially trained to self-administer either drug under a FR-1 schedule of reinforcement (as described in chapter 2) until they acquired instrumental responding (significantly higher level of responding on the active lever than on the inactive lever thereby showing discrimination and stable rate of injections). Following acquisition of instrumental learning, rats were then exposed to Fixed Interval schedules with increasing durations from 1 minute (FI-1) to 15 min (FI-15). They were trained to seek either drug for three days under the FI15 schedule of reinforcement after which they were exposed to 21 daily sessions of heroin seeking under a SOR, as described in chapter 2 (**Fig. 6.3**). Forty-five min after a drug-free 15 min challenge session during which they responded under the control of CSs, presented contingently upon every 10th lever press, brains were harvested (as described in chapter 2). Tissue was and processed to assess DAT protein levels in protein pools extracted from micro punches from several identified striatal territories (heroin, n=10; cocaine, n=12) or from cultured astrocytes (heroin, n=10; cocaine, n=11) from the same regions as those used for the micro punches.

In order to better understand if the changes in DAT protein levels in each striatal territory predate the recruitment of aDLS dopamine-dependent mechanisms, thereby testing if these were adaptations to drug exposure more than a neurobiological correlate of the expression of incentive habits, rats (n=40) were subjected to cocaine (n=17), heroin (n=11), or food (n=12) self-administration under a FR-1 schedule of reinforcement, as previously discussed.

Rats from cocaine and heroin groups were maintained under FR-1 schedule of reinforcement for as long as they needed to reach the same total number of drug infusions as those trained under SOR. Rats trained to self-administer food pellets were maintained under FR- 1 schedule of reinforcement (as described in chapter 3) until they acquired instrumental responding and

displayed stable levels of responding (**Fig. 6.3**).

In order to better compare these samples to those obtained from the SOR groups while avoiding the acute pharmacological effects of the drugs and potential drug-withdrawal effects, brains were harvested 22 hours following the last self-administration session (as described in chapter 2) and processed to assess DAT protein levels in protein samples from micro punched striatal territories (heroin, n=5; cocaine, n=6 and food pellet, n=6) or cultured astrocytes (heroin, n=6; cocaine, n=11 and food pellet, n=6). Brains from naïve rats (n=12) were also harvested and processed into striatal micro punched structures (n=6) or cultured astrocytes (n=6) to control for a potential modulation of DAT protein levels by instrumental learning (when compared to food

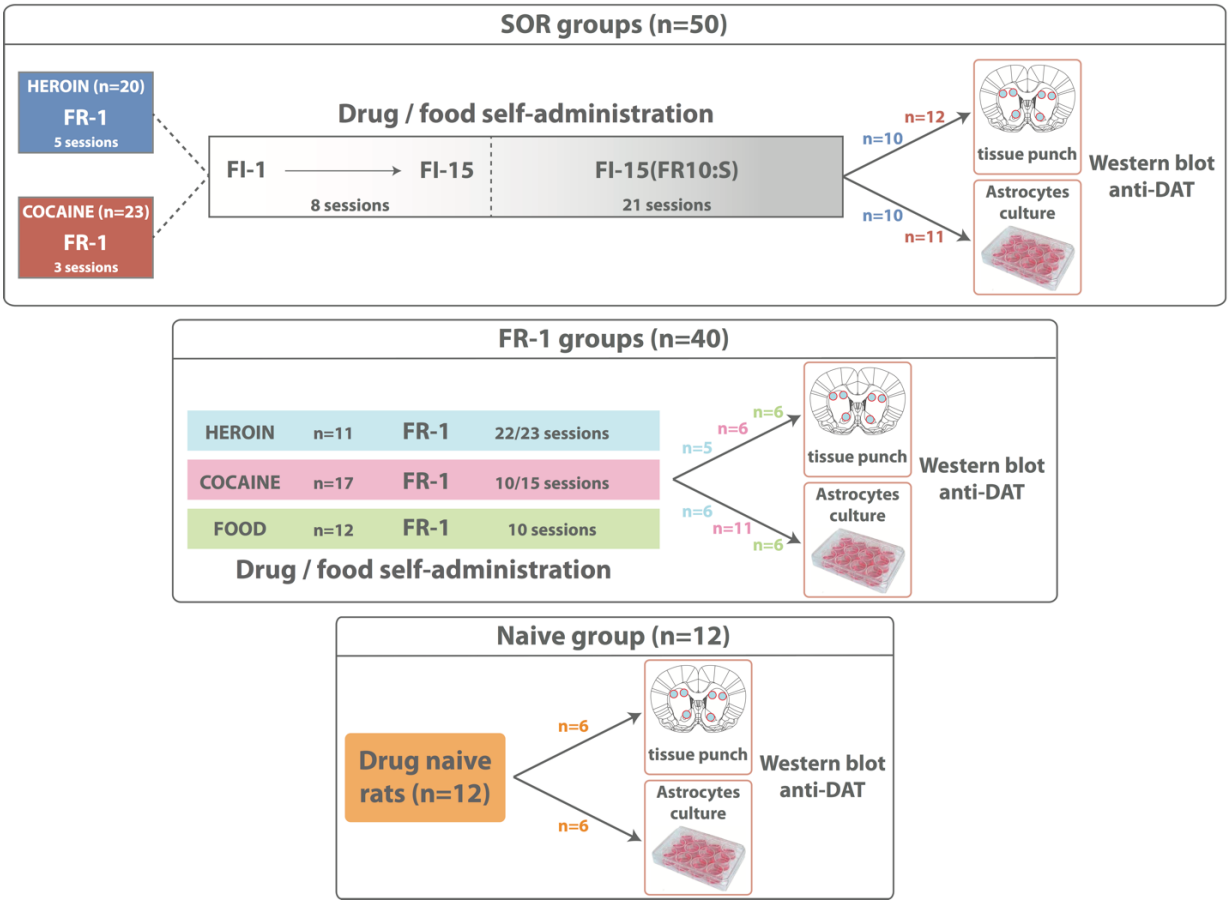


Figure 6.3: Schematic representation of the experimental design. *Rats were trained to self-administer either cocaine or heroin under a FR-1 schedule of reinforcement, then under fixed interval schedules eventually to be maintained under SOR for 21 days. Other cohorts of rats were trained to self-administer cocaine, heroin or food under FR1 until they reached the same total number of infusions as displayed by those maintained under SOR. A group of naïve rats was also included in the study in order to understand the direction of the changes observed in drug- or food-exposed rats. Following a drug-free seeking challenge test, brains were harvested and processed either to conduct primary astrocytes culture from striatal areas or to micro-punch these striatal areas. DAT protein levels were then assessed by western-blot assays from the micro-punched structures (containing proteins from both neurons and astrocytes as well as the other CNS cells) and from primary astrocytes cultures.*

controls (**Fig. 6.3**).

2. Micro punches

Brains were processed into 300 µm-thick coronal sections as described in chapter 2 and striatal territories, namely, aDLS, aDMS, pDLS, pDMS and AcbC were bilaterally punched from different brain sections as shown in **Fig. 6.4**. Punched samples were weighed and mixed with lysis buffer (10µl /mg of sample), protein lysates were stored at -20ºC until further use.

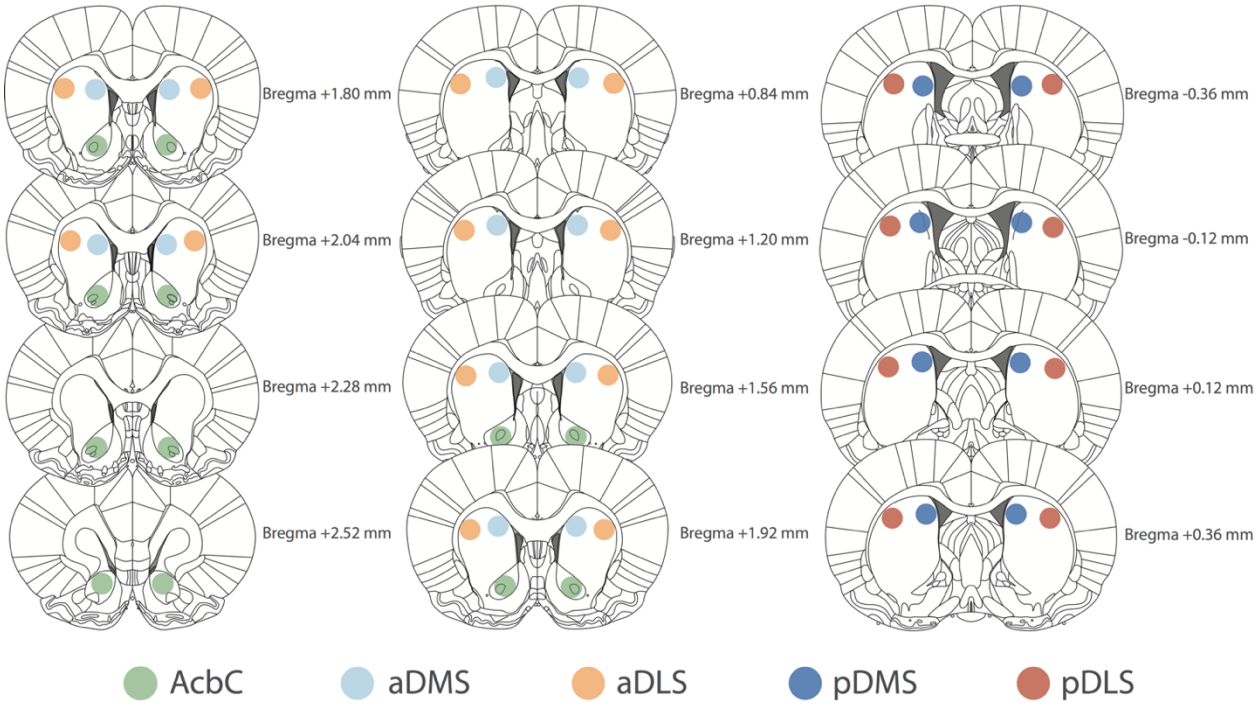


Figure 6.4: Schematic representation of the localisation of micro-punched and dissected striatal territories. *From [395].*

3. Astrocytes primary culture

Brains were dissected and cells cultured as described in chapter 2. Following 4 to 6 weeks of culture, the only cells present were astrocytes. They received lysis buffer as described in chapter 2 and protein lysates were stored at -20ºC until western blot assays.

To validate the relevance of the cell culture methods and confirm the exclusive presence of astrocytes, striatal cells from naïve rats were cultured as described in chapter 2 and co-immunostained for GFAP (astrocyte marker [475]) and Iba-1 (microglial marker [476]). This validation was carried out twice alongside the astrocyte culture from each experimental group.

Samples from freshly dissected brains were dissociated as described in chapter 2 and cell suspensions were plated on poly-D-lysine (*Sigma Aldrich*) coated coverslips. Cells were cultured for 4 to 6 weeks as described in chapter 2, then coverslips were washed 3 times with cold PBS 1X (4°C) during 5 min and cells were fixed with 500 µl of 4% PFA during 20 min. Coverslips were washed 3 times with cold PBS 1X (4°C) for 5 min. A blocking solution (1X PBS, 2% BSA (*Santa Cruz*), 1% Triton X-100 (*Sigma Aldrich*)) was applied to coverslips for two hours at room temperature. GFAP primary antibody (*Merck Millipore, #04-1062*) hosted in rabbit, was diluted at a concentration of 1/500 in the blocking solution. 40 µl of primary antibody solution were dropped on a parafilm in a wet chamber and coverslips were flipped over so that cells entered in contact with the solution. The wet chamber was hermetically closed and stored at 4°C for an overnight incubation.

The following day, coverslips were washed 3 times with cold 1X PBS (4°C) for 5 min and Iba-1 primary antibody (*Merck Millipore, #MABN92*) hosted in mouse was diluted at a final concentration of 1/1000 in the blocking solution. Coverslips were flipped over on a 40 µL drop of this solution in a wet chamber and stored at 4°C for an overnight incubation, as previously described.

For the first assessment, coverslips were washed 3 times with cold 1X PBS (4°C) during 5 min and anti-rabbit secondary antibody conjugated with Alexa Fluor® 647 (*Merck Millipore, #AP187SA6*) was diluted at a concentration of 1/800 in the blocking solution. Cells were incubated with the secondary anti-rabbit antibody for 90 min at room temperature in the wet chamber; coverslips were then washed 3 times with 1X PBS for 5 min.

Anti-mouse secondary antibody conjugated with Alexa Fluor® 488 (*Merck Millipore, #AP124JA4*) was diluted at a concentration of 1/800 in the blocking solution and incubated with cells at room temperature for 90 min in the wet chamber.

For the second assessment, anti-rabbit secondary antibody conjugated with Alexa Fluor® 488 and anti-mouse secondary antibody conjugated with Alexa Fluor® 647 were used at the concentrations of 1/800.

Following 3 washes (1X PBS, 5 min), 50 µl of the nucleus marker DAPI (*Life Technology, #D1306*) were applied on cells for 4 min at room temperature. Coverslips were washed 3 times

(1X PBS, 5 min), and then mounted on slices with drops of DPX mounting medium (*Fischer, #D/5319/05*). Slices were let to dry overnight in a dark at room temperature.

Signals were captured by a camera (*Qimaging*) mounted on a fluorescence microscope using a 20X magnification objective and acquired images were treated using *Qcapture* software.

4. Western blot assays

The consecutive steps of western blot assays, i.e. sample lysis, electrophoresis, transfer of proteins onto nitrocellulose membranes, incubations of membranes with antibodies and signal detection, were carried out as described in chapter 2.

The primary antibody anti-DAT, hosted in rabbit, was custom-made by Dr. Bruno Giros' lab and was used at the concentration of 1/750. Beta-actin was used as loading control protein and the primary antibody anti-beta actin (*Abcam, ab8226*) hosted in mouse was used at the concentration of 1/75000 to target this protein. Secondary antibodies were HRP linked (enabling ECL detection), i.e. anti-rabbit (*cell signalling, 7074S*) and anti-mouse (*ImmunoReagents Inc. gtxmu-003-dhrpx*) and were both used at the final concentration of 1/1000.

5. Data and statistical analyses

The protein of interest (DAT) / control protein (actin) ratio was calculated and used as dependent variable. Since it was non-normally distributed as revealed by the Sapiro-Wilk's test a linear transformation (2+Log(x)) was applied and the resulting variable passed the normality test.

The overall experimental design was not symmetrical and the sample size of the various experimental groups used in the analysis of the DAT protein level across various striatal territories and between treatments was relatively small. Thus, for each structure analysed, upon verification of a main group effect, subsequent post-hoc analyses were carried-out based on *a priori* hypotheses using planned comparisons [477] that rely on contrast analysis whereby specific drug-exposed groups only were systematically compared to drug naïve groups, irrespective of their training history, as previously described [262].

Results

1. Behavioural training

Rats from the SOR-cocaine and SOR-heroin conditions acquired drug self-administration under a FR-1 schedule of reinforcement over a period of 3 and 5 days, respectively, as revealed by a higher level of responding on the active lever as compared to the inactive lever *[SOR-cocaine: main effect of lever: $F(1,21) = 123.24$, $p < 0.0001$; session: $F(2,42) < 1$; and lever x session interaction: $F(2,42) = 7.741$, $p < 0.01$; SOR-heroin: main effect of lever: $F(1,18) = 5.279$, $p < 0.05$; session: $F(4,72) = 1.065$, $p > 0.05$; and lever x session interaction: $F(4,72) = 4.705$, $p < 0.01$]*. No difference was observed between the rats that were subsequently allocated to the "punch" (micropunches) or the "astro" (astrocyte culture) groups *[SOR-cocaine: main effect of group: $F(1,21) = 2.775$, $p > 0.1$ and group x lever interaction: $F(1,21) = 6.784$, $p < 0.05$; SOR-heroin: main effect of group: $F(1,18) = 1.724$, $p > 0.05$ and group x lever interaction: $F(1,18) < 1$]* (data not shown).

Rats were then trained to seek cocaine or heroin under fixed interval schedules with increasing interval duration over daily sessions, which resulted in a robust increase in active lever presses. No differences were observed between the punch and astro groups in their acquisition of drug self-administration and drug seeking (i.e. from FR-1 to FI-15 schedules of reinforcement) *[SOR-cocaine: main effect of group: $F(1, 21)=1.265$, $p>0.1$, session: $F(10,210) = 35.630$, $p < 0.0001$; and session x group interaction: $F(10,210) < 1$; SOR-heroin: main effect of group: $F(1, 18) < 1$, session: $F(12,216) = 62.782$, $p < 0.0001$; and session x group interaction: $F(12,216) < 1$]*(**Fig. 6.5 A** & **D**).

Introduction of the CSs, presented contingently upon every tenth lever press resulted in an increase in active lever presses, persistent over sessions that was similar between punch and astro groups *[SOR-cocaine: main effect of group: $F(1, 21) \leq 1$, session: $F(23,483) = 11.157$, $p < 0.0001$; and session x group interaction: $F(23.483) < 1$; SOR-heroin: main effect of group: $F(1,18) \leq 1$, session: $F(23,414) = 11.715$, $p < 0.0001$; and session x group interaction: $F(24,414) < 1$]*, suggesting that drug seeking behaviour invigorated by CSs acting as reinforcers was instantiated as an incentive habit (**Fig. 6.5 A** & **D**).

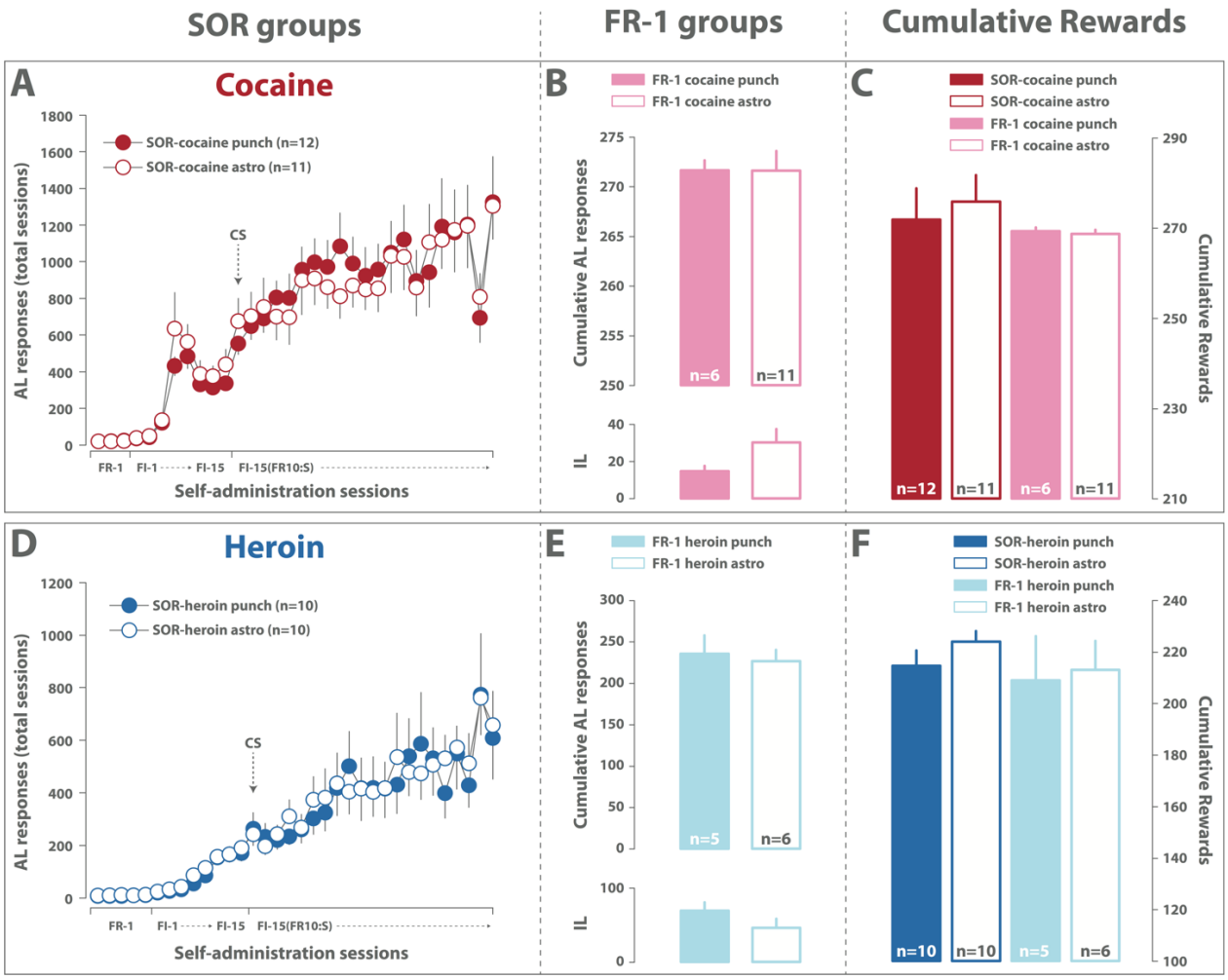


Figure 6.5: Behavioural characterisation of drug-experienced groups. *A and D) Rats trained to self-administer cocaine or heroin under SOR acquired and maintained high levels of drug seeking behaviour under the control of conditioned stimuli. No difference was observed between punch and astro groups in their level of responding. B and E) Rats trained to self-administer drugs under a FR-1 schedule of reinforcement acquired drug self-administration similarly across the various conditions and experimental groups. C and F) The total number of infusions received in rats exposured to cocaine or heroin was matched between the reinforcement schedules.*

Rats maintained under FR1 schedule of reinforcement for cocaine or heroin (FR1-cocaine and FR1-heroin, respectively) acquired drug self-administration similarly irrespective of which group (punch vs astros) they were subsequently allocated to. Thus, no differences were observed between the punch and astros group trained under continuous reinforcement for cocaine or heroin in their allocation of responding to the active lever *[FR1-cocaine: main effect of lever: $F(1,15) = 4194.1$, $p < 0.0001$; group: $F(1,15) = 1.535$, $p > 0.1$; and lever x group interaction: $F(1,15) = 4.093$, $p > 0.05$; FR1-heroin: main effect of lever: $F(1,9) = 103.97$, $p < 0.0001$; group: $F(1,9) = 1.539$, $p > 0.1$; and lever x group interaction: $F(1,9) < 1$]* (**Fig. 6.5 B** & **E**).

All the rats trained under continuous reinforcement for cocaine or heroin received the same number of drug infusions as those with an history of drug self-administration under SOR for cocaine and heroin, respectively *[cocaine conditions: main effect of group: F(3,36) < 1; heroin conditions main effect of group: F(3,27) < 1]* (**Fig. 6.5 C** & **F**).

Rats trained to instrumentally respond for food pellets under a FR-1 schedule of reinforcement for 5 daily sessions rapidly acquired the contingency and discriminated early on in training the active and inactive levers *[Punch group: main effect of lever: F(1,5) = 111.19, p < 0.0001 ; session: F(9,45) = 6.5747, p < 0.0001 ; and lever x session interaction: F(9,45) = 8.0918, p < 0.0001; astro group: main effect of lever: F(1,5) = 2893.2, p < 0.0001 ; session: F(9,45) = 18.772, p < 0.0001 ; and lever x session interaction: F(9,45) = 18.885, p < 0.0001]* (**Fig. 6.6**).

No difference was observed between the rats that will be allocated to the punch or the astro group *[main effect of group: F(1,10)=3.974, p>0.05 and group x lever interaction: F(1,10)=7.0121, p<0.05]*.

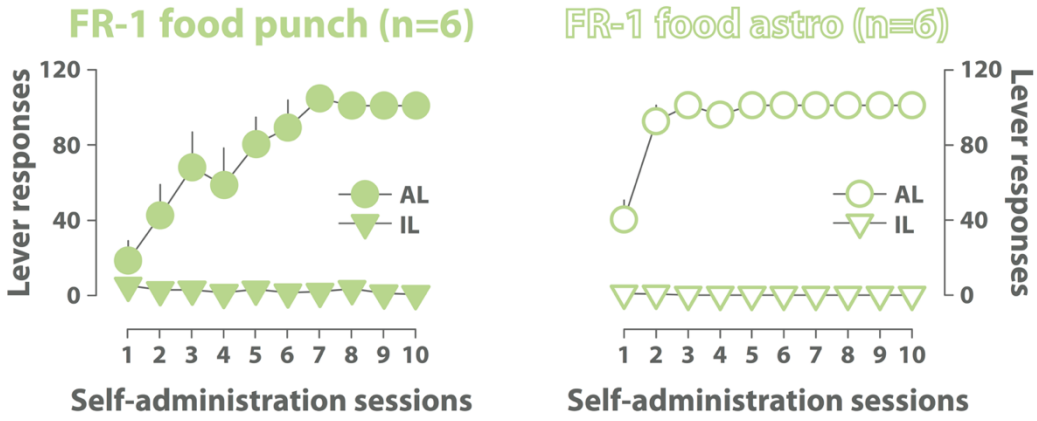


Figure 6.6: Behavioural characterisation of the groups trained to respond instrumentally for food. *Rats trained to self-administer food pellets under a FR-1 schedule of reinforcement for 10 days acquired an almost absolute discrimination between the active and inactive lever by the fifth session. No differences were observed between the punch and astro groups.*

2. Primary astrocytes culture

Following 4 to 6 weeks of culture, cells presented the specific anatomical characteristics of cultured astrocytes with a slender shape and long processes (**Fig. 6.7A**). Co-immunostaining of GFAP and Iba-1 conducted twice, revealed that all cells labelled by DAPI were GFAP-positive and Iba-1-negative, thereby confirming the exclusive presence of astrocytes, with no microglial contamination (**Fig. 6.7B** & **C**).

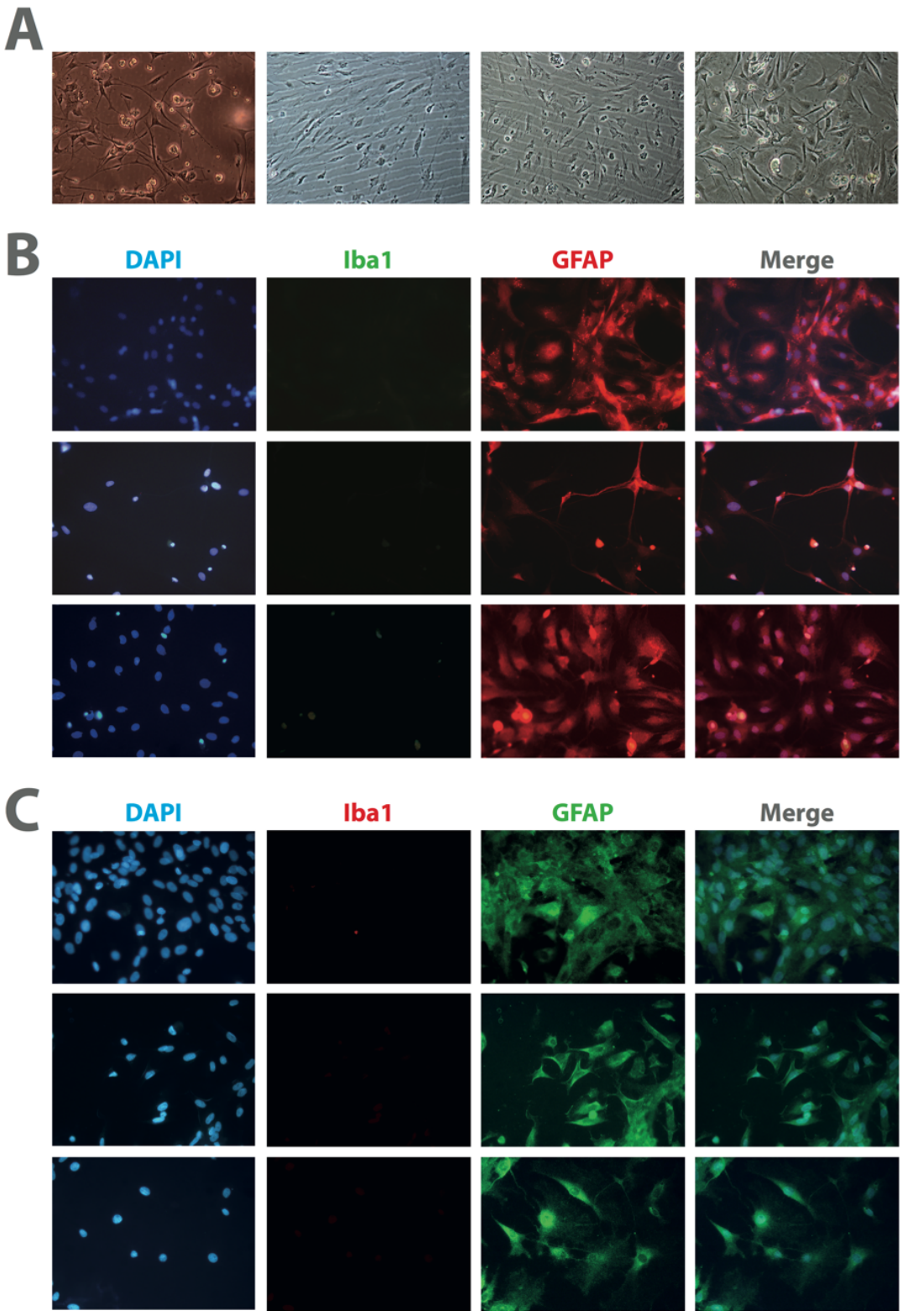


Figure 6.7: Characterisation of the cell populations observed after several weeks of culture. ***A)*** *Pictures of cultured cells in bright field. Cells presented the specific anatomical features of cultured astrocytes (slender shape and long processes).* ***B)*** *Immuno-localisation of GFAP and Iba1 were performed on 4 to 6 weeks old primary astrocytes cultures. Cells which were labelled in blue by DAPI (nucleus) were GFAP-positive and Iba1-negative, confirming the relevance of the cell culture method.*

3. Striatal DAT protein levels

DAT protein levels were assessed by western blot assays from the micro-punched striatal structures and from the primary astrocytes cultures obtained from the same striatal territories.

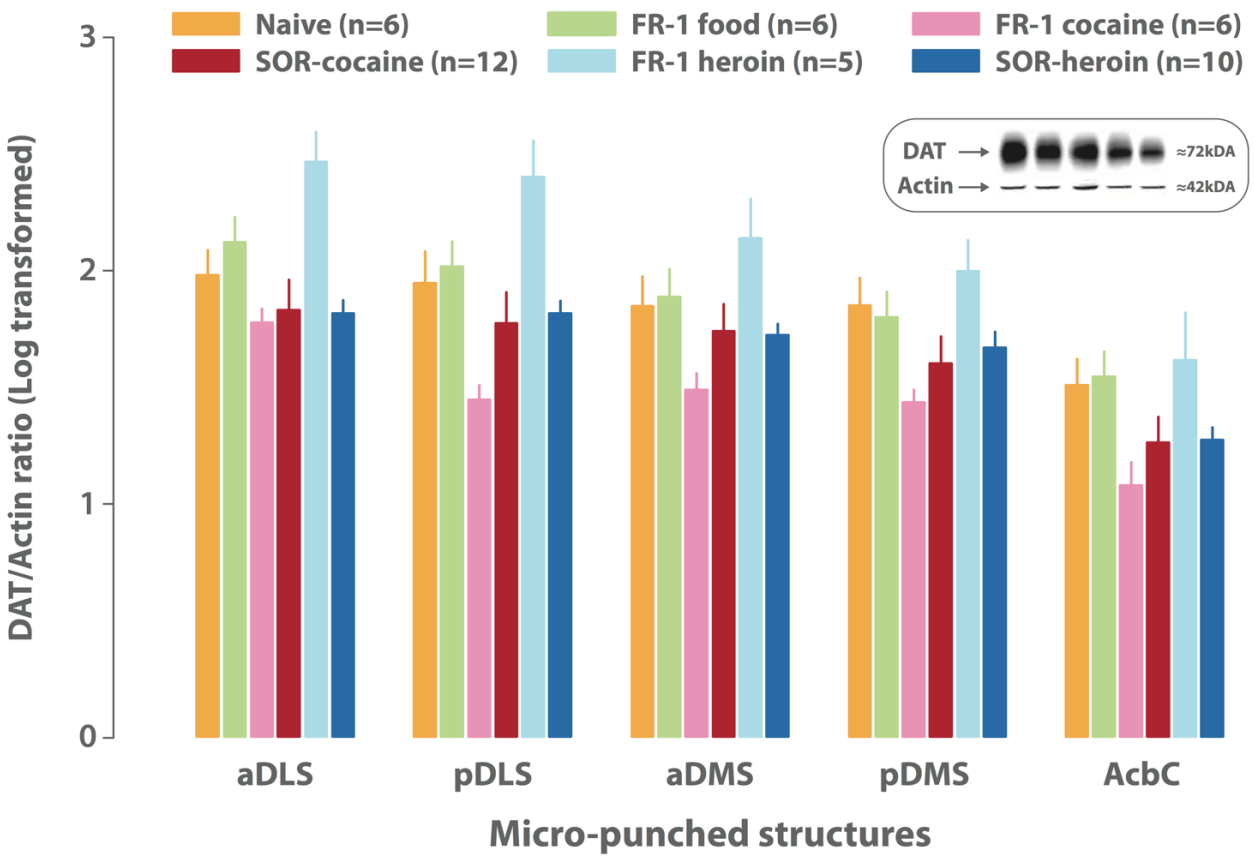


Figure 6.8: DAT protein levels in striatal micro-punched structures*. The level of expression of the DAT protein was differentially distributed through the different territories of the striatum following a ventrodorsal gradient. Rats trained to self-administer cocaine (both FR1 and SOR groups) and heroin (only SOR group) displayed a decrease in DAT protein levels in all the striatal territories as compared to control groups (naïve and FR-1 food).Rats trained to self-administer heroin under a FR-1 schedule of reinforcement displayed a higher DAT protein expression as compared to control groups. Insert: illustration of the nature of the signal obtained after western-blot of the target (DAT) and cotnrol (Actin) proteins.*

When all the striatal structures were considered together, DAT protein levels appeared to be differentially distributed along the striatal territories following a ventro-dorsal gradient [main effect of *structure: $F(4,148) = 100.52$, $p < 0.0001$]*. Exposure to either cocaine or heroin, irrespective of the nature of the reinforcement schedule, resulted in a decrease in DAT protein levels across the striatal territories, at the exception of the FR1-heroin group which displayed an increase DAT protein levels expression as compared to the control groups, i.e. naïve and FR1-food groups (for which DAT protein levels were identical) *[main effect of group: $F(5,37) = 4.257$,*

p < 0.01; and group x structure interaction: F(20,148) = 1.623, p > 0.05] (**Fig. 6.8**). Since the FR1-heroin group clearly behaved as an outlier here, the quantitative nature of the drug-induced decrease in DAT protein levels in each of the striatal territory was analysed using planned comparisons testing the hypothesis that all drug-exposed groups, excluding the FR1-heroin group, would display an overall decrease in DAT levels as compared to drug-naïve groups, irrespective of their training history.

Thus, in each of the striatal territories, upon confirmation of a significant main effect of group (considering all experimental groups, including FR1-heroin as between-subject factors), a contrast analysis was carried out to compare the DAT levels of all the rats of drug exposed groups (excluding the FR1-heroin group) to those of the drug naïve groups (**Table 6.1**).

Structures	Main group effect			Contrast analysis Drug Vs No-drug		Contrast analysis SOR Vs No-drug	
	Degrees of freedom	**F** values	*p* values	**F** values	*p* values	**F** values	*p* values
aDLS	1,38	4.7377	<0.01	5.5931	<0.05	4.6213	<0.05
pDLS	1,37	5.5293	<0.001	7.3394	<0.05	2.5822	ns
aDMS	1,37	2.8848	<0.05	4.2668	<0.05	1.5668	ns
pDMS	1,38	2.8311	<0.05	6.4297	<0.05	3.3239	ns
AcbC	1,38	2.8696	<0.05	9.1739	<0.01	5.5446	<0.05

Table 6.1. Main effects and statistical outcome of planned contrast analyses on DAT levels in each of the striatal territories. *Left column represents the statistical outcome of the main group effect taking into account all the experimental groups as between-subject factor. Upon verification of a main effect of group, contrast analyses were performed specifically to test the hypothesis that drug exposed groups, excluding FR1-heroin, displayed a decrease in DAT protein levels in each striatal territory as compared to all the drug-naïve rats, both in the naïve and FR1-food conditions (middle comlumn). Another series of independent contrast analsyses was carried out comparing only the SOR groups to the drug naïve groups in order to bettter characterise the adaptations specific to incentive habits.*

Interestingly, when the incentive habit groups only where contrasted to the drug naïve groups, differences in the DAT protein levels were only observed in the aDLS and the AcbC, but not in the DMS or the pDLS any longer, thereby revealing that potential adaptations may occur differentially in the AcbC-aDLS functional network that underlies the development of incentive habits (**Table 6.1**).

The down-regulation of DAT observed in micro-punched striatal structures (containing both neurons and astrocytes) from drug-experienced rats (apart from the FR-1 heroin group) could be explained by the total blunting of DAT expression in astrocytes from the same territories,

assessed following primary cultures (**Fig. 6.9**). Indeed, even if miss-represented in **Fig 6.9** for clarity, no signal whatsoever, under any potential detection condition tested, was visualised for the astrocytes cultured from the drug-exposed rats. Thus, in the absence of quantitative value and of variance, these dramatic changes were not subjected to statistical analysis. Interestingly, the complete loss of DAT expression in striatal astrocytes was also observed in those of the FR1-heroin.

In marked contrast astrocytes from naïve and FR1-food groups expressed DAT proteins and did not differ from each other, even if the FR1-food group tended to display a slight decrease (albeit non statistically significant) in DAT protein levels as compared to the naïve group in the dorsal striatal territories *[main effect of group: $F(1,5) = 1.036$, $p > 0.1$; structure: $F(4,20) = 1.126$, $p > 0.1$; and group x structure interaction: $F(4,20) < 1$]* (**Fig. 6.9**).

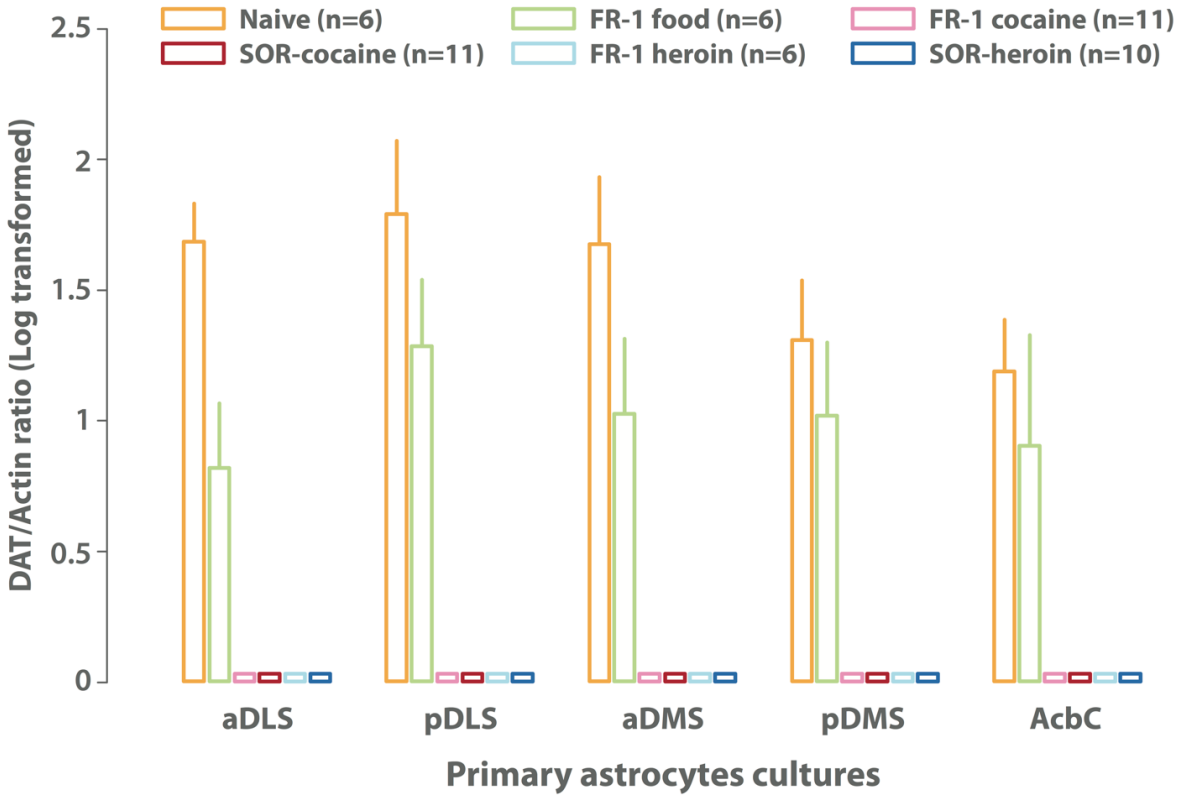


Figure 6.9: DAT protein levels in cultured astrocytes from striatal territories. *No difference was observed in DAT protein levels between naïve and FR1-food groups across the different striatal areas. However, DAT proteins were not detected on in astrocytes from drug-experienced groups.*

Discussion

This study aimed to better characterise the adaptations in DAT protein levels within whole tissue and astrocytes from different striatal territories over the course of the establishment of incentive habits for cocaine, and heroin.

At the behavioural level, all rats trained to self-administer either cocaine or heroin under a SOR acquired and maintained high levels of drug seeking under the control of conditioned stimuli, thereby confirming the robustness of the procedure, even in Sprague Dawley rats. Rats trained to self-administer drugs or natural rewards under continuous reinforcement acquired instrumental responding rapidly and their training was controlled so that their overall drug intake (for the drug SA groups only) matched the one displayed by rats with an history of 21 days of training under SOR.

Brains harvested from the astro groups of each experimental condition as well as from naïve rats were processed for primary astrocytes cultures which was successfully set-up and validated in the lab since following 4 or 6 weeks of culture, the wells contained exclusively astrocytes as revealed by co-immunostaining of GFAP and IBA-1.

As anticipated from the literature, at least in humans [478], and from the results of chapter 5, DAT protein levels decreased in response to drug exposure in all the examined striatal territories. Independent contrast analyses further revealed that these drug-induced decrease in striatal DAT protein levels were only present in rats showing incentive habits, both for heroin and cocaine, in the aDLS and the AcbC. This is, I believe, a very interesting result as it suggests that the direction and magnitude of DAT adaptations triggered by exposure to cocaine, and, to some extent, heroin (see subsequent discussion of the FR1-heroin group) may evolve over long-term training under a SOR, when intoxication levels are rather low, in striatal areas that are not functionally involved in the expression and maintenance of an incentive habit.

This work identified that the decrease in striatal DAT protein levels occurred in incentive habits only within the AcbC-aDLS functional unit which was previously identified as being their core neural locus [146] and shown to have aberrant internal functional coupling in former heroin addicts [416].

This overall conclusion is mitigated by the unexpected observation that rats trained to self-administer heroin under a FR-1 schedule of reinforcement displayed an up-regulation of DAT expression as compared to control groups. While the decision was made to report this result, it warrants a replication experiment as, for practical and experimental reasons, the western blot assay and association quantification of that group was analysed separately from the other experimental groups and this difference may well represent a change in the experimental conditions under which the independent assays were carried out. Nevertheless, if confirmed, this difference does indeed suggest that opposite trajectories occur between cocaine and heroin between the early response to heavy intoxication and long-term adaptation to cue-controlled drug seeking as both drugs trigger a decrease of similar distribution across and magnitude within the different striatal territories. Thus, it could be hypothesised that the differential early adaptations to chronic cocaine and heroin self-administration may reflect the sensitivity of the expression level of DAT to the spiking activity of dopaminergic neurons. Indeed, while cocaine blocks DAT and enhances activity-dependent concentrations of dopamine, heroin triggers a disinhibition of dopaminergic neurons. This differential response could also reflect a direct influence of heroin onto μ-OR-mediated mechanisms within the striatum. Clearly further research is required to better understand these data.

The decrease in DAT levels observed in proteins extracted from micro-punched striatal territories of drug-experienced rats (apart from FR1- heroin group) relative to drug naïve animals may be entirely attributable to the complete loss of DAT in astrocytes. Indeed, the striking result from this study is that DAT proteins that were expressed on striatal astrocytes from naïve rats or rats trained instrumentally to respond for food could not be detected in any of the striatal territories of the drug experienced groups, irrespective of the drug or the training history, including the FR1-heroin group.

This blunting of DAT protein levels in striatal astrocytes occurred similarly in rats exposed to cocaine and heroin. This observation suggests that the physical interaction between cocaine and DAT and the associated altered function of the latter are not the cause of the downregulation of the protein levels. The observed decrease also cannot be accounted for a decrease in the membrane expression of DAT since total protein levels were studied here. As detailed in the

general introduction, cocaine and heroin, and more broadly, drugs of abuse, trigger hyperdopaminergic states primarily in the ventral striatum, but would actually artificially increase dopamine concentrations at any dopaminergic terminal in which dopamine clearance is mediated by DAT. Therefore, it can be concluded that this downregulation in DAT protein levels in astrocytes is consecutive to a chronic dopaminergic state brought about by exposure to either cocaine or heroin. In contrast with the results from the qPCR experiments of chapter 5, which revealed that SOR-cocaine and SOR-heroin groups showed differential astrocytic levels of DAT mRNA in the ventral striatum a complete loss of the DAT protein was observed here to be similar between these groups. This suggests that the adaptations leading to the total blunt of astrocytic DAT are more likely to take place around the translational and post-translation modifications of the protein DAT. Nevertheless, the cellular mechanisms whereby hyperdopaminergic states result in the downregulation of DAT expression in astrocytes are not understood but they could involve mechanisms downstream of the cellular activation of astrocytes or directly by dopamine taken up into the cytoplasm by DAT [472], or else by differential activation of dopaminergic receptors located on the cell membrane of astrocytes [471]. Astrocytic adaptations to hyperdopaminergic states could also involve functional interactions with dopaminergic or post-synaptic neurons in the striatum. These neurons also respond to hyperdopaminergic states by releasing neuronal messengers, such as endocannabinoids, adenosine or NO which directly influence the activity of astrocytes [468, 470], and may, consequently, trigger down-regulation of DAT. Since both heroin and cocaine also result in a decrease in GLT1, which is remediated by NAC, further research is necessary to understand whether these observations are downstream similar mechanisms and if NAC also remediates the downregulation of DAT. This would offer further insight into whether the regulation of glutamate and dopamine homeostasis is coupled in astrocytes in the striatum.

The complete loss of DAT protein expression in striatal astrocytes occurred both in the ventral and dorsal territories of the striatum, even in the FR1-cocaine and FR1-heroin groups, in which aDLS dopamine-dependent mechanisms had not been recruited [202]. This suggests that adaptations in the mechanisms that control synaptic dopamine homeostasis in the aDLS predate its functional recruitment and associated maladaptive drug seeking habits. Thereby astrocytes

may contribute to the dopamine-dependent mechanisms involved in the intrastriatal functional shifts subserving the development of compulsive drug seeking habits. The observation of a complete loss of DAT proteins in astrocytes of the FR1-heroin group is at odds with the apparent increase in total DAT, further supporting the need for a replication of that experiment.

At the circuit level, since the drug-induced loss of DAT in astrocytes impinge on their potential capacity to buffer extra synaptic dopamine, it can be hypothesised that it results in a dysregulation of dopamine volume transmission. Thus, beyond the aberrant increase in synaptic and extra synaptic dopamine concentration triggered by administration of cocaine or heroin, the levels of dopamine released in response to the CRs, as observed in the aDLS of rats engaged in cue-controlled cocaine seeking under a SOR [479], could eventually spill outside de vicinity of the synapse. This could eventually lead to recruitment of adjacent territories of the striatum, and facilitate the functional coupling of different striatal territories. Such a hypothesis is supported by the anatomical organisation of the dopamine-dependent intrastriatal circuitry. In both rats and non-human primates, ventral striatal territories are functionally coupled with more dorsal territories via their reciprocal projections to the mesencephalon. Spiralling connections between striatal regions and midbrain dopaminergic neurons (located in the VTA and substantia nigra) offer an ascending functional circuitry within the striatum [415, 480] in which each striatal region can influence dopaminergic mechanisms in its latero-dorsal neighbour. Since the functional recruitment of aDLS dopamine-dependent cocaine seeking habits has been shown to be dependent on this circuitry [146, 481], the alterations in astrocytes revealed here could be considered to facilitate functional recruitment of adjacent striatal territories from the ventral to the dorsolateral striatum over the course of drug seeking history. In this context, astrocytes may further contribute to a dopamine-dependent functional coupling of striatal territories, all of which show the loss of astrocytic DAT expression, through an interconnected network, often referred as behaving like a syncytium [482, 483]. Thus, modulation in intracellular calcium levels triggered by dopamine could be large enough to create calcium waves spreading to adjacent and more distant astrocytes, thereby offering a mechanism whereby ventral and dorsolateral striatal neurons become controlled by a network of astrocytes.

Further comparing the differential pattern of decrease in DAT protein levels within astrocytes

and the entire tissue, it is important to further consider that the complete loss of expression of DAT in astrocytes not only occurred in rats with an history of continuous reinforcement, which seemed to be the conditions under which the DAT protein levels decreased the most in the whole tissue across striatal territories, but also in rats with an history of incentive habits for heroin and cocaine. Thus, the outcome of the contrast analyses that revealed that in the SOR condition, the decrease in total DAT protein levels as compared to drug naïve rats was only statistically significant in the aDLS and the AcbC, needs to be compared with the complete loss of astrocytic DAT expression in all the striatal territories. This suggests that the recovery of DAT protein decrease in the other striatal territories (i.e. aDMS, pDMS, pDLS) may be attributable to an upregulation of DAT expressed in the terminals of dopamine neurons. Thus, while adapting to the loss of function of the astrocytic DAT, these seemingly spared striatal territories of rats with an incentive habit may be subjected to a complete different dopamine transmission. If the extra-synaptic astrocytic DAT has not actually recovered from the acute effects of continuous reinforcement, but is compensated for by an increase in the expression of presynaptic DAT on dopamine neurons, it may be the case that the temporal properties of phasic, as well as tonic, dopamine transmissions have reached an allostatic state that may contribute to a decrease in response to reinforcers and associated cues that are not related to the drug. Further research is necessary to better test this hypothesis.

Four to six weeks of culture may, by itself, trigger modifications in protein expression levels in astrocytes that are not controlled for in the present experiment. Yet, the differences observed cannot be solely accounted for by the culture conditions since astrocytes harvested from structures from rats trained to self-administer drugs are compared to those control or drug-naive rats. Nevertheless, in order further to validate these observations DAT expression could be assessed from neurons and astrocytes extracted and sorted out directly from freshly dissected brains by Fluorescent-Activated Cell Sorting (FACS). Thus, follow up experiments should be carried out in which Adeno-associated viruses (AAV) carrying the open reading frame of GFP or mCHERRY under a NeuN-1 or GFAP promoter, respectively, would be injected into different striatal areas so that neurons and astrocytes would be labelled with GFP or mCHERRY, respectively. Rats would then be trained to self-administer cocaine and heroin under the same

schedules as those used in this study. Striatal samples would then be assessed by FACS to segregate neurons (expressing GFP) and astrocytes (expressing mCHERRY). Then, DAT protein or mRNA levels could be quantified in these two cell populations by western blot or qPCR, respectively.

The results of this investigation open many new questions, and several other experiments to be carried out, including:

1. teasing apart the contribution of the bi-directional communication between neurons and astrocytes, from a direct effect of dopamine on the astrocytic expression of DAT.
2. Testing the potential impact of cocaine and heroin on cultured astrocytes calcium signalling using calcium imaging assays (and/or ratiometric calcium measurements).
3. Testing the influence of causal chemogenetic inhibition/activation of astrocytes in the ventral vs dorsolateral striatum on the development and expression of incentive habits and underlying neurobiological correlates as identified in chapter 5.

Nevertheless, these results presented in this chapter suggest a causal contribution of astrocytes in the intrastriatal shift subserving the development of incentive habits. They are, I believe, ground-breaking in that they challenge completely our, or at least my, understanding of the intrastriatal mechanisms involved in the development of drug addiction.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION

The main objective of this PhD research project was to identify inter-individual differences in the vulnerability to develop compulsive heroin seeking habits and identify their psychological, behavioural, neural and cellular basis. That was indeed a very ambitious endeavor that required to implement a vertical top-down strategy from experimental psychology to cellular and molecular investigations of characterized neural networks. Having had to set-up both our behavioural facility and wet laboratory twice in the course of this PhD, once in the Department of Pharmacology and once in the Department of Psychology has posed a real challenge to successfully complete the experiments that were initially planned. Nevertheless, a novel procedure was developed in the rat to measure individual differences in the vulnerability to develop compulsive heroin seeking habits.

This novel model of compulsive heroin seeking habits whereby drug seeking during the second half of each 15-min interval of a FI15(FR10:S) second order schedule of reinforcement results in contingent deliveries of mild electric foot shocks has great heuristic value and construct validity with regards to the human situation. Indeed, in real life drug addicts behaviourally manifest the compulsive nature of their behaviour while they are engaged in foraging for their drugs over protracted periods of time, in a drug-free state. This drug seeking behaviour that progressively becomes engrained as a maladaptive habit, is not divorced from the motivational value of Pavlovian cues, in that drug addicts eventually rely on cues in the environment, acting as conditioned reinforcers, to bridge delays to reinforcement and invigorate ongoing instrumental responding.

In the procedure developed here, the compulsive nature of heroin seeking was successfully operationalised to fulfil all these characteristics: (i) only 20% of the individuals with an history of heroin use (identified with an unbiased cluster analysis strategy) demonstrated a persistence of responding for heroin in the face of current, future or past punishment, (ii) that persistence of instrumental responding in the face of punishment was displayed in rats which drug seeking response was not only under the control of aDLS-dependent S-R associations, but was also intimately associated with, and reinforced by, the drug-paired conditioned stimuli acting as

conditioned reinforcers. (iii) the compulsive nature of heroin seeking was measured over several minutes daily in the absence of the drug, and therefore cannot be attributable to the analgesic properties of heroin.

The newly established procedure of compulsive heroin seeking habits enabled the parametric investigation of the influence of the extended exposure to conditioned reinforcement, previously hypothesized to result in incentive habits [7, 144, 145], on the sensitivity of the heroin seeking response to punishment. Thus, punishment as introduced in our procedure, seemed to have the same influence on heroin seeking behaviour in both SOR and FI-15 conditions, thereby suggesting that incentive habits do not contribute to compulsivity, at least when compulsivity is defined as resistance to contingent punishment. However, incentive habits were shown here specifically to facilitate the expression of maladaptive drug seeking behaviour in anticipation of, and following, punishment. This anticipation of the inability to respond due to negative consequences during punished periods of time reflects an aberrant drive to express the instrumental response at a time when it is not appropriate to do so since rats had learnt over several sessions not to engage in vigorous responding during the first half of each interval. Thus, in the face of a future loss of opportunity to freely respond, rats showing incentive habits engage in aberrant responding beforehand, as well as once punishment is terminated.

This phenomenon that is not merely the reflecting of S-R control over behaviour, as it is not displayed by rats with an history of training under a FI15 schedule of reinforcement, has a great heuristic value with regards to the human condition wherein individuals addicted to drugs engage in aberrant drug seeking behaviour despite the knowledge of future negative consequences, or despite having endured negative consequences in the past. Consequently, the present data, alongside research carried-out on forced abstinence for cocaine by Dr. Yolanda Pena-Oliver (unpublished) offers evidence that overtraining under SOR results in behavioural and psychological adaptations to instrumental deprivation, under either forced or voluntary abstinence, are very different to those observed after training under fixed interval schedules of reinforcement. These adaptations, which cannot be accounted for only differences in rate of responding, offer an objective delineation of the nature of incentive habits, which stem from the protracted reinforcement and invigoration of a S-R controlled instrumental response, as it is the

case under fixed interval schedules of reinforcement, by contingent presentations of drug-paired CSs acting as conditioned reinforcers.

Overall these results warrant critical re-appraisal of the definition of compulsivity and its operationalisation in animal models and to revisit the theoretical framework within which the relationship between habits and compulsivity is understood. Compulsivity should perhaps be redefined as the aberrant engagement in drug seeking behaviour during, but also before and after punishment.

Of marked interest, the development of compulsive cocaine seeking, which behavioural characteristics are similar to those observed for heroin, was observed in vulnerable individuals after only 17 days of training under SOR and a total of ~ 300 infusions. This is in marked contrast with the evidence that not a single compulsive rat was identified out of a rather large cohort of rats self-administering cocaine for 21 days under extended access [315], representing more than 2200 infusions, or that the 3 criteria phenotype emerges in rats self-administering cocaine for 3 hours per day after 85 sessions, representing as well more than 2000 infusions [174, 192, 193, 212]. This reveals that drug intoxication is necessary for the development of compulsive behaviour in vulnerable individuals, but not sufficient, and that the psychological mechanisms engaged in the drug seeking response, are more important in that process than previously acknowledged.

In the search for behavioural markers of vulnerability to develop compulsive heroin seeking habits, I relied on the power of longitudinal studies whereby cohorts of rats are characterised for several behavioural traits prior to drug exposure and are retrospectively analysed as being predictive of the transition to compulsivity either at the between-subject or dimensional level [174, 192, 212, 267, 484].

None of the behavioural dimensions and associated traits predicted the propensity to acquire heroin self-administration under continuous reinforcement, thereby demonstrating that the reinforcing properties of heroin, at least at the doses tested here (it is important to keep in mind that HR rats differ from LR rats in their intake of cocaine only at low doses, but not at the one used in the present studies for example [192]). Since these were exploratory experiments which represent a massive personal and financial investment, the decision was made to use the dose

of heroin that had been shown previously to support high and stable levels of responding under a SOR. However, future research will be necessary to investigate behavioural traits of increased sensitivity to the reinforcing properties of heroin.

Despite the relative low statistical power yielded from the otherwise carefully planned experimental design, high anxiety trait and to a lesser extent, sign tracking were identified as potential predictive markers of individual vulnerability to develop compulsive heroin seeking habits. High anxiety trait, which had been previously shown in the lab not to predict escalation of heroin self-administration [209], predicted a marked increase in motivation for heroin when rats were trained under fixed interval schedules of reinforcement, at the same dose for which no differences were observed under continuous reinforcement, as discussed above. HA rats also persisted in responding for heroin in a drug-free state longer than LA rats, clearly demonstrating that a high anxiety state influences the initial motivation for heroin and subsequent development of compulsive heroin seeking habits. This observation offers direct support to the self-medication hypothesis of opiates addiction [107, 275, 485] and suggests that the psychoaffective state and associated motivation (negative as opposed to positive reinforcement) under which the individual experiences the drug may facilitate the transition to addiction. If HA rats acquire heroin self-administration to cope with an internal distress, their apparent higher motivation under fixed interval schedules may be similar to the one displayed by animals experiencing the rewarding properties of heroin under withdrawal [486] thereby learning about the increased motivational value of heroin once it has alleviated withdrawal. This observation would also fit very well within the remit of the hedonic allostasis theory [111, 141] which is, after all, an acquired self-medication strategy in response to the between-systems adaptations triggered by heroin, and especially the recruitment of the central stress system, suggested to contribute to a switch from positive to negative reinforcement. In this context, the drug-induced recruitment of between-systems adaptations would develop on an already negative reinforcement process and would thereby aberrantly strengthen in these vulnerable individuals.

However, there is an alternative, yet, complimentary interpretation of these findings. Since all addictive drugs eventually recruit the stress system and that stress, and likely anxiety, facilitates DLS control over behaviour [487, 488], HA rats may have developed a more engrained habitual

control over behaviour. Under the conditioned reinforcing properties of the CSs during training under SOR, they may have developed a stronger incentive habit than LA rats since the CS would be imbued with higher incentive motivational properties. This is clearly a risky speculation but it could be quite easily tested in subsequent experiments, in which HA rats treated with an anxiolytic prior to, and during exposure to heroin, should display diminished behavioural features of addiction for heroin. Finally, whether external stressors, prior to drug exposure or indeed while HA and LA rats are engaged in cue-controlled drug-seeking would influence the vulnerability or resilience to display high motivation for heroin and compulsive heroin seeking would be another avenue for future research since opiates have been shown to influence the responsivity to stressors [489, 490].

At the neural and cellular level, this work has identified a clear differential pattern of functional recruitment of corticostriatal structures between incentive habits and habits, but not between compulsive and non-compulsive rats. The hotspot analysis carried out using in situ hybridisation targeting zif268 mRNA has been a successful strategy to investigate the differential recruitment of functional neural networks in rats expressing drug seeking as a habit, an incentive habit or a compulsive incentive habit. The analysis of neuronal plasticity throughout the entire corticostriatal circuitry offered unprecedented insights into the neural substrates of incentive habits and compulsive heroin seeking behaviour. Incentive habits for heroin were shown to recruit cellular plasticity in the same brain regions as those previously identified to be supporting incentive habits for cocaine [146, 245], thereby suggesting a common neural basis of incentive habits for the two drugs. Additional analysis suggested that incentive habits for heroin do not stem from a deficient goal-directed system as compared to heroin seeking habits (as it has been suggested for other conditions [417]), but instead from an aberrant engagement of subcortical Pavlovian and S-R instrumental mechanisms [145]. In addition, various correlations highlighted the importance of the interaction between the intoxication history of the individual and the recruitment of a specific psychological mechanism, and associated neurobiological substrates.

Thus, this work offers compelling evidence at the behavioural, psychological and neural level that responding under a SOR does not just reflect the expression of a drug seeking habit, the magnitude of the behavioural expression of which is increased by CRs, but reflects the

development of a distinct mechanism, so called incentive habit, as initially hypothesised by Belin and colleagues [7, 144, 145].

The investigation of molecular and cellular correlates within the different territories of the striatum revealed distinct neurobiological adaptations associated with the expression of compulsivity for heroin and cocaine, highlighting the potential importance of the interactions between adenosinergic and dopaminergic mechanisms in the ventral striatum. Even if similar neural networks seem to be recruited for incentive habits for cocaine and heroin (as previously mentioned), the association between the drug and the expression of incentive habits triggered differential neurobiological adaptations in the adenosinergic and dopaminergic systems throughout the striatum.

Further investigations of the transcriptomic profile of the structures of the corticostriatal circuitry are warranted to better understand the nature of the cellular basis of incentive habits and compulsive heroin seeking. Additionally, direct comparison with drug naïve animals would be an optimal strategy further to probe whether the observed adaptations reflect down- or upregulations as compared to baseline and whether these differences also predate the drug exposure.

Finally, some of these adaptations were found to be attributable to astrocytes rather than neurons as revealed by the down-regulation of astrocytic dopamine transporter following either cocaine or heroin exposure. This suggest that, extracellular levels of dopamine brought about by drug exposure would triggered astrocytic adaptations which could lead to recruit and functionally control entire striatal territories. Therefore, this would potentially trigger the recruitment of adjacent, dorsal, striatal territories and a dopamine-dependent functional coupling of the ventral and dorsolateral striatum, facilitating the development of incentive habits. Further investigations of the poorly described role of astrocytes within the dopaminergic tripartite synapse are warranted to better understand the role of this cell population in the development of compulsivity and incentive habits.

These studies offered new insights in the behavioural endophenotypes of vulnerability to compulsive heroin seeking habits. Added to the wealth of data in the literature on endophenotypes of vulnerability to cocaine addiction, these data highlight the importance of the

nature of the reinforcer and the psychological mechanisms recruited while intoxication occurs, in the pathophysiology of addiction, in which astrocytes clearly play a more prominent role than previously considered.

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