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Cocaine addiction in the rat: alterations in brain functions and novel medications

Referees: Prof. Dr. Rainer Spanagel
Prof. Dr. Thomas Kuner

List of Papers

This thesis contains data from papers (I-III) below.

I. Vengeliene V*, **Takahashi TT***, Dravolina OA, Belozertseva I, Zvartau E, Bespalov AY, Spanagel R. (2018) Efficacy and side effects of baclofen and the novel GABAB receptor positive allosteric modulator CMPPE in animal models for alcohol and cocaine addiction. Psychopharmacology (Berl). 2018 Jul;235(7):1955-1965.

II. Cannella N, Cosa-Linan A, Roscher M, **Takahashi TT**, Vogler N, Wängler B, Spanagel R. (2017) [18F]-Fluorodeoxyglucose-Positron Emission Tomography in Rats with Prolonged Cocaine Self-Administration Suggests Potential Brain Biomarkers for Addictive Behavior. Front Psychiatry. Nov;1; 8:218.

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IV. Vengeliene V, Roßmanith M, **Takahashi TT**, Alberati D, Behl B, Bespalov A, Spanagel R. (2018) Targeting Glycine Reuptake in Alcohol Seeking and Relapse. J Pharmacol Exp Ther. Apr;365(1):202-211.

V. Vengeliene V, Cannella N, **Takahashi T**, Spanagel R (2016) Metabolic shift of the kynurenine pathway impairs alcohol and cocaine seeking and relapse. Psychopharmacology 233(18):3449-59.

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List of Abbreviations

0crit	cocaine non-addicted like
3crit	cocaine addicted-like
4P-PDOT	4-Phenyl-2-propionamidotetralin
5-HT	serotonin
^{18}F	fluorine atom
^{18}F-FDG	^{18}F-fluorodeoxyglucose
AC	age control
Acb/NAc	nucleus accumbens
ACC	anterior cingulate cortex
AD	axial diffusivity
AMPA	alphaamino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMY	amygdala
ANOVA	analysis of variance
AP	active nose-pokes
Au	auditory cortex
BLA	basolateral amygdala
BOLD	blood oxygenation level dependent
BP	break point
CeA	central amygdala
Cg1	cingulate cortex 1
Cg2	cingulate cortex 2
CMRGlu	cerebral metabolic rate of glucose
CMPPE	2-{1-[2-(4-chlorophenyl)-5-methylpyrazolo[1,5-a]pyrimidin-7-yl]-2-piperidinyl}ethanol
CNS	central nervous system
CPu	caudate putamen
CRF	corticotropin-releasing factor
CS	conditioned stimulus
CSA	cocaine self-administration
CSF	cerebrospinal fluid
DA	dopamine
dlPFC	Dorsolateral prefrontal cortex
DLT	dorsolateral thalamus
DMSO	dimethyl sulfoxide
DP	dorsal peduncular nucleus

drug-ON	drug availability periods
DS	dorsal striatum
DSM-5	Diagnostic and Statistical Manual of Mental Disorders
DTI	diffusion tensor imaging
E-SARE	enhanced-SARE Promotor
Ent	entorhinal cortex
EPI-FID	echo-planar imaging - free induction decay
ext	extinction
FA	fractional anisotropy
FAST	FMRIB's Automated Segmentation Tool
FD	frame-wise displacement
FDR	false discovery rate
fMRI	functional magnetic resonance imaging
FR	fixed ratio
FrA	frontal association cortex
FWHM	full-width at half-maximum
GABA	gamma-aminobutyric acid
GIRK	G-protein coupled inwardly rectifying potassium
GP	globus pallidum
GDP	gross domestic product
GMV	grey matter volume
CMRGlu	cerebral metabolic rate of glucose
GPi	globus pallidus or entopeduncular nucleus in rodents
HcPD	posterodorsal hippocampus
HcSDG	hippocampus subiculum and dentate gyrus
HcV	hippocampus ventral
Hyp	hypothalamus
i.p.	intraperitoneally
ICD-10	the International Statistical Classification of Diseases and Related Health Problems 10^{th} revision
IEGs	immediate early genes
IHC	immunohistochemistry
IL	infralimbic cortex
IP	inactive nose-pokes
LD	longitudinal diffusivity
LTD	long-term depression
LTP	long-term potentiation
M2	secondary motor cortex

MD	mean diffusivity
MDT	midline dorsal thalamus
mGluR2/3	metabotropic glutamate inhibitory autoreceptors
mPFC	medial prefrontal cortex
MRI	magnetic resonance imaging
MSNs	medium spiny neurons
MT_2	N-[(4-methoxy-1H-indol-2-yl)methyl]propanamide
NA	noradrenaline
Nac	nucleus accumbens core
NaS	nucleus accumbens shell
NMDA	N-methyl-D-aspartate
NMR	nuclear magnetic resonance
NO-drug	non-availability of drug
NOS	nitric oxide synthase
OF	open field
OFC	orbifrontal cortex
PAG	periaqueductal gray
PET	positron emission tomography
PFA	paraformaldehyde
PFC	prefrontal cortex
PIT	Pavlovian-instrumental transfer
PL	prelimbic cortex
Prl	prelimbic region
PSD	postsynaptic density
RARE	rapid acquisition with refocused echoes
RD	radial diffusivity
Reinst	reinstatement test
RF	radiofrequency electromagnetic radiation
ROIs	regions of interest
RS	retrosplenial cortex
Rs-fMRI	resting state functional magnetic resonance imaging
s.c.	subcutaneous
S-R	stimulus–response
S2	secondary somatosensory cortex
SA	self-administration
SC	superior colliculus
SCN	suprachiasmatic nucleus

Sept	septum
SN	substantia nigra
SUD	substance use disorders
SUV	standardized uptake values
SUVR	average of SUV within the entire brain mask that excludes regions outside the brain
TBSS	tract-Based Spatial Statistics
TIFF	tagged image file format
TPM	tissue probability maps
TR/TE	repetition time/echo time
Tu	olfactory tubercle
VBM	voxel-based morphometry
Veh	vehicle
VI	variable interval
VMT	ventromedial thalamus
VP	ventral pallidum
VTA	ventral tegmental area
WM	white matter of cerebellum
ZI	zona incerta

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Summary

Cocaine addiction is a chronic mental illness affecting a small subgroup of cocaine users (approx. 18%). Little efforts have been taken so far to understand individual differences in the vulnerability to cocaine addiction; i.e. it is unclear why some users become addicted whereas the majority of them are able to maintain the control over drug-taking and -seeking. Most preclinical studies have not considered the possibility to study individual differences in addiction vulnerability and furthermore may explain the high failure rates of drugs that reach Phase III-IV of clinical trials. Hence, a translational animal model is fundamental to produce meaningful results to the clinic. In this thesis, a DSM-IV/5-based preclinical model of cocaine addiction was used to identify pre-existing vulnerability differences and the changes produced by pathological state of addiction. The so-called 0/3crit animal model consists of training rats for intravenous cocaine self-administration for at least 45 sessions. Thereafter, three main DSM-based addiction behavioral criteria were tested, (1) motivation to take the drug, (2) persistence to seek the drug, and (3) persistence of self-administration despite negative consequences (i.e., application of an electric foot-shock). For each of the three criteria, a score of either 1 or 0 was given to animals performing above or below 60th percentile of the population distribution, respectively. Animals positive for all criteria (3crit) were classified as addicted-like, whereas animals negative for all criteria (0crit) were classified as non-addicted-like/resilient rats.

This thesis had three aims:

(i) Neuroimaging studies in cocaine addicts revealed various structural and functional changes but findings are inconclusive and this may be due to pre-existing features in the function or structure in brains of vulnerable subjects and different drug intake patterns. Based on this, longitudinal studies, particularly in controlled animal models, are warranted. Here a longitudinal translational multimodal neuroimaging study was performed using the 0/3crit animal model of cocaine addiction to investigate pre-existing differences and changes in brains of cocaine addicted-like and non-addicted-like rats.

(ii) Conditioned cocaine cues can trigger craving and relapse. Pavlovian conditioned stimuli can also impact ongoing instrumental behavior, even if the instrumental behavior is acquired independently of Pavlovian conditioning. This process is called Pavlovian-to-Instrumental transfer (PIT). A hypothesis in drug addiction posits that Pavlovian conditioned cues can bias instrumental behavior towards drug seeking and intake and that a more pronounced PIT response occurs in vulnerable subjects. The second aim was therefore to test this hypothesis and to ask whether 3crit rats show a more pronounced PIT response than 0crit rats.

(iii) Pharmacological treatments in cocaine addiction are still lacking. In previous preclinical studies different glutamate receptor antagonists and $GABA_B$ agonists such as baclofen, have been tested but either failed in clinical studies or reported severe side effects in pharmacovigilance. Here, different pharmacological approaches were taken: First, the novel $GABA_B$ positive allosteric modulator CMPPE was tested in comparison to baclofen in order to produce lesser side effects. Second the NR3A subunit was selectively targeted as an alternative to NMDAR antagonists. Finally, melatonin was tested for normalizing altered circadian rhythmicity in cocaine addiction.

The longitudinal MRI results revealed structural changes in the brains of 3crit and 0crit rats. Grey matter (GM) volume was found to have increased in prelimbic (Prl) and cingulate (Cg) cortices, nucleus accumbens (NAc), caudate putamen (CPu), substantia nigra, and ventral and globus pallidum in the 3crit group, whereas 0crit rats showed no changes in GM volume, except for the CPu, compared to control. Diffusion tensor imaging (DTI) analysis revealed a higher fractional anisotropy in the zona incerta in the 0crit rats compared to 3crit rats. PET results showed higher activity of mPFC and right CPu in the 0crit group compared to controls. Arc expression was significantly reduced in the infralimbic (IFL) cortex in the 3crit group. Salience of conditioned-cues was found similar in 0crit and 3crit rats in the PIT paradigm. Positive allosteric modulator of $GABA_B$ receptors and melatonin abolished cue-elicited cocaine-seeking behavior.

In summary, cocaine addiction produced structural changes in brain regions central for motivation and drug rewarding effects in 3crit rats, whereas the addiction resilient rats showed increased volume in brain regions involved in habit behavior as well as an increased in microstructural integrity in a brain area that regulates adaptive behavior. Functional assessments indicated the relevance of the mPFC (Prl and IFL) activity for both controlled or compulsive drug-seeking and –taking. These results agree with clinical studies, where mPFC function negatively correlates with impulsive behavior in psychostimulant abusers as well as changes were found in brain regions, such as Prl, Cg, CPu, and NAc, that are known to play a role in drug addiction. Moreover, other factors, such as structural and functional changes, instead of Pavlovian or instrumental conditioning may lead to addiction behaviors because salience to conditioned stimuli and learning ability were similar between the groups. And finally, positive allosteric modulator of $GABA_B$ receptors and melatonin appears to be promising candidates for medication development in cocaine addiction. The 0/3crit model of cocaine addiction has excellent face validity and can be used to study the underpinning mechanisms that lead to compulsive drug use.

Zusammenfassung

Kokainsucht ist eine chronische psychische Erkrankung, die eine kleine Untergruppe der Kokainkonsumenten betrifft (ca. 18%). Bisher wurden kaum Anstrengungen unternommen, um individuelle Unterschiede in der Anfälligkeit für Kokainsucht zu verstehen. Es ist unklar, warum einige Konsumenten süchtig werden, während die Mehrheit von ihnen in der Lage ist, die Kontrolle über Drogeneinnahme und -suche zu behalten. Die meisten präklinischen Studien haben diese individuellen Vulnerabilitätsunterschiede nicht beachtet, was die hohen Ausfallraten der Medikamente erklären könnte, welche die Phasen III-IV von klinischen Studien erreichen.
Daher ist ein translationales Tiermodell von grundlegender Bedeutung, um aussagekräftige Ergebnisse für die Klinik zu liefern. In dieser Arbeit wurde ein DSM-IV / 5-basiertes präklinisches Modell der Kokainabhängigkeit verwendet, um bereits bestehende Vulnerabilitätsunterschiede und die durch den pathologischen Zustand der Abhängigkeit hervorgerufenen Veränderungen zu identifizieren. Das so genannte 0 / 3crit-Tiermodell beruht auf einem Training für Ratten zur intravenösen Kokain-Selbstverabreichung für mindestens 45 Tage. Anschließend wurden drei Haupt-DSM-basierte Suchtverhaltenskriterien getestet: (1) Motivation, die Droge zu nehmen, (2) die Beharrlichkeit, nach der Droge zu suchen, und (3) Fortbestehen der Selbstverabreichung trotz negativer Konsequenzen (die Gabe eines elektrischen Fuß-Schocks). Für jedes der drei Kriterien wird ein Wert von 1 oder 0 an Tiere vergeben, die oberhalb oder unterhalb des 60. Perzentils der Populationsverteilung liegen. Tiere, die für alle Kriterien positiv waren (3crit), wurden als suchtähnlich eingestuft, während Tiere, die für alle Kriterien (0crit) negativ waren, als nicht suchtähnliche/ resiliente Ratten klassifiziert wurden.

Diese Doktorarbeit hatte drei Ziele:

(i) Neuroimaging-Studien bei Kokainsüchtigen zeigten verschiedene strukturelle und funktionelle Veränderungen, aber die Befunde sind nicht schlüssig, was auf bereits vorhandenen Merkmalen in der Gehirnfunktion oder -struktur von anfälligen Personen und unterschiedlichen Drogeneinnahmemustern zurückzuführen sein kann. Auf dieser Grundlage sind longitudinale Studien, insbesondere in kontrollierten Tiermodellen, gerechtfertigt. Hier wurde eine longitudinale translationale multimodale Neuroimaging - Studie mit Hilfe des 0 / 3crit - Tiermodell der Kokainsucht verwendet, um vorab bestehende Unterschiede und Veränderungen im Gehirn von Kokain - abhängigen und nicht - abhängigen Ratten zu untersuchen.

(ii) Konditionierte Stimuli können Verlangen nach Kokain und den Rückfall auslösen. Pawlowsche konditionierte Stimuli können sich auch auf das anhaltende instrumentelle Verhalten auswirken, auch wenn das instrumentelle Verhalten unabhängig von der Pawlowschen Konditionierung erworben wird. Dieser Prozess wird als Pawlowschen-zu-instrumentellen-Transfer (PIT) bezeichnet. Eine Hypothese der Drogenabhängigkeit postuliert, dass Pawlowsche konditionierte Stimuli eine Tendenz vom instrumentellen Verhalten hin zur Suche und Einnahme von Drogen bewirken können und dass bei gefährdeten Personen eine stärker ausgeprägte PIT-Reaktion auftritt. Das zweite Ziel war daher, diese Hypothese zu überprüfen und zu fragen, ob 3crit Ratten eine stärkere PIT-Reaktion als 0crit Ratten zeigen.

(iii) Noch immer fehlen pharmakologische Behandlungsmöglichkeiten der Kokainsucht. In früheren präklinischen Studien wurden verschiedene Glutamat-Rezeptor-Antagonisten und $GABA_B$-Agonisten wie Baclofen getestet, die entweder in klinischen Studien fehlschlugen oder bei denen schwere Nebenwirkungen in der Pharmakovigilanz berichtet wurden. Hier wurden verschiedene pharmakologische Ansätze verfolgt: Zunächst wurde der neue $GABA_B$-positive allosterische Modulator CMPPE im Vergleich zu Baclofen getestet, um die Nebenwirkungen zu reduzieren. Zweitens wurde die NR3A-Untereinheit gezielt als Alternative zu NMDAR-Antagonisten ausgewählt. Schließlich wurde Melatonin auf eine Normalisierung der veränderten zirkadianen Rhythmik bei Kokainabhängigkeit getestet.

Longitudinale MRT-Ergebnisse zeigten strukturelle Veränderungen in den Gehirnen von 3crit- und 0crit-Ratten. Es wurde ein erhöhtes Volumen der grauen Substanz (GM) im prälimbischen (Prl) und cingulären (Cg) Kortex, dem Nucleus accumbens (NAc), Caudate Putamen (CPu), Substantia nigra und ventral und globus pallidum in der 3crit Gruppe gefunden, während 0crit Ratten im Vergleich zur Kontrollgruppe keine Veränderungen im GM Volumen zeigten, mit Ausnahme des CPu. Die Diffusions-Tensor-Bildgebungs (DTI) Analyse zeigte eine höhere fraktionelle Anisotropie in der Zona incerta bei den 0crit-Ratten im Vergleich zu den 3crit-Ratten. PET-Ergebnisse zeigten eine höhere Aktivität des mPFC und dem rechten CPu in der 0crit-Gruppe im Vergleich zur Kontrollgruppe. Die Arc-Expression war im infralimbischen (IFL)-Kortex in der 3-Crit-Gruppe signifikant reduziert. Eine ähnliche Salienz konditionierter Stimuli wurde bei 0crti und 3crit Ratten im PIT Paradigma gefunden. Das Stimulus-induzierte Kokainsuchverhalten wurde durch die Gabe des positiven allosterischen Modulators der $GABA_B$-Rezeptoren und Melatonin aufgehoben.

Zusammenfassend wurden durch Kokainabhängigkeit in 3crit Ratten strukturelle Veränderungen in Hirnregionen induziert, die zentral an der Motivation und der belohnenden Wirkung der Droge beteiligt sind. Die Sucht-resilienten Ratten zeigten ein erhöhtes Volumen in Hirnregionen, welche in Gewohnheitsverhalten eine Rolle spielen. Des Weiteren zeigten diese Tiere eine erhöhte mikrostrukturelle Integrität einer Hirnregion, welche in der Regulation adaptiven Verhaltens eine Rolle spielt. Funktionelle Auswertungen zeigen die Relevanz der mPFC (Prl und IFL) Aktivität für kontrolliertes oder zwanghaftes Drogensuchverhalten und Drogeneinnahme. Diese Ergebnisse stimmen mit klinischen Studien überein, bei denen die mPFC-Funktion negativ mit dem impulsiven Verhalten bei Psychostimulanz-Konsumenten korreliert. Zudem stimmen die Ergebnisse damit überein, dass Regionen wie Prl, Cg, CPu und NAc bekanntermaßen eine Rolle bei der Drogenabhängigkeit spielen. Darüber hinaus könnten andere eher Faktoren, wie strukturelle und funktionelle Veränderungen anstatt Pawlowsche oder instrumentelle Konditionierung zu Suchtverhalten führen, da die Salienz gegenüber konditionierten Reizen und die Lernfähigkeit zwischen den Gruppen ähnlich ist. Schließlich scheinen der positive allosterische Modulator von $GABA_B$-Rezeptoren und Melatonin vielversprechende Substanzen für die Entwicklung neuer Medikamente zur Behandlung von Kokainsucht zu sein. Das 0 / 3crit-Modell der Kokainabhängigkeit hat eine exzellente Augenscheinvalidität und kann bei der Identifizierung der Mechanismen helfen, welche den zwanghaften Verhaltensweisen der Drogensuche und der Drogeneinnahme unterliegen.

Chapter 1: General Introduction

Substance use disorders (SUD) cause high economic costs to governments and devastating consequences to life of drug users and their close ones. Worldwide it is estimated that 275 million people worldwide, age spanning from 15 to 64 years old, have used drug once in 2016 and 31 million of them are regular drug users with SUD, which means their drug use is detrimental and treatments may be needed (WHO, 2018). Overdose or SUD-related deaths are common, and about 450 thousand people lost their lives in 2015. In Europe, United Kingdom (31 %) and Germany (15 %) together account for about half of the overdose deaths. Regarding costs, European countries have spent an estimated 0.01 % to 0.5 % of gross domestic product (GDP) in drug-related public expenditure (EMCDDA, 2017). Germany alone spent around ~ 0.25 % of their GDP, corresponding to EUR 5.2 and 6.1 billion in 2006 (EMCDDA, 2017). Alarming is the fact that the drug market is expanding. Data on cocaine specifically indicates 25 % increase in coca cultivation as well as in cocaine consumption from 2015 to 2016 (WHO, 2018).

1.1 Cocaine

Cocaine or benzoylmethyl ecgonine is an alkaloid from the coca plant *(Erythroxylon coca*). It was isolated in the 1850's by German chemist Albert Niemann (Johanson and Fischman, 1989) and its purified form (Fig. 1) was popularized in the medical community and used as local anesthetic and vasoconstrictor for few decades (Barash, 1977). Sigmund Freud, who used the drug himself, referred it as a 'magical substance' and cure for several physical and psychological illnesses. However, the abusive potential of cocaine was soon discovered and control laws were introduced in early 1900's. In 1914 cocaine was banned and its consumption decreased until the 1960's. By the popularization of crack cocaine, its consumption increased again and became a substantial public health problem.

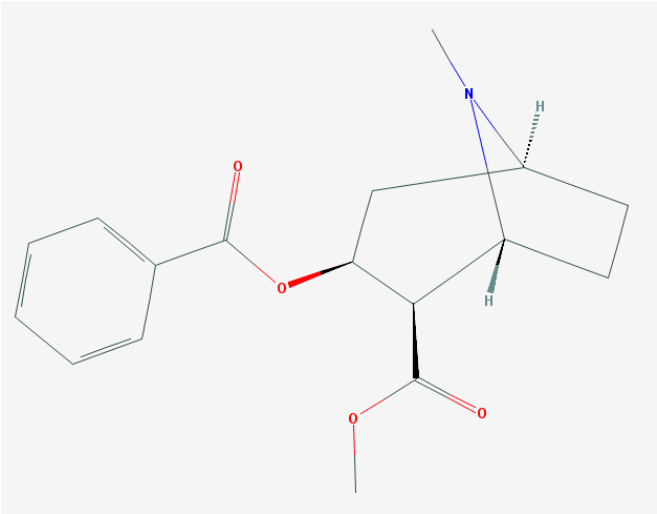


Figure 1 Chemical structure of cocaine
Cocaine is an alkaloid ester, with molecular formula $C_{17}H_{21}NO_4$. Image acquired from Pubchem, Open Chemistry Database, 2018.

Cocaine is classified as a stimulant of central nervous system (CNS) and its effects include euphoria, alertness, suppression of the appetite, increased feelings of pleasure and increased locomotor activity. It can also produce anxiety, paranoia and restlessness. Cocaine is a weak basic substance and can exist as water-soluble salts, or in a free base form ('crack cocaine'), which is inhaled via smoking. Different to amphetamines that has a chemical structure resembling noradrenaline and dopamine, cocaine's structure resembles other synthetic local anesthetics and thus, its use as a topic anesthetic. When smoked or intravenously injected, cocaine concentration peaks within 5 to 10 min, while it takes up to 60 min after intranasal administration (Cone, 1995). The 'high' produced by cocaine typically lasts for 30 to 60 min, and its half-life is roughly 1h (Inaba, 1989; Jufer et al., 2000; Moolchan et al., 2000). Part of cocaine is excreted unchanged in the urine, but the majority is metabolized to inactive forms of cocaine, including benzoylecgonine, ecgonine methyl ester, and norcocaine (Jufer et al., 2000; Klingmann et al., 2001).

1.2 Brain Reward Pathway and drug addiction

The discovery of reward-related brain areas by Olds and Milner (1954) significantly affected our understanding of brain circuitry. The researchers found that rats implanted with electrodes in regions rich in dopaminergic cell bodies produced repeated lever pressing for intracranial self-stimulation, suggesting that stimulation of these dopaminergic regions is reinforcing (Corbett and Wise, 1980; Olds and Olds, 1963; Wise, 1981). Dopamine (DA) neurons project rostrally from the substantia nigra and ventral tegmental area (VTA) to release DA in brain structures involved in motivation and goal-directed behavior, such as dorsal striatum (DS), nucleus accumbens (NAc) and frontal cortex, known as mesocorticolimbic dopaminergic pathway (Fig. 2). Activation of this projection signals the importance of the stimuli that are critical for survival and therefore, natural reinforcers, such as food, water and sex, also activate this pathway.

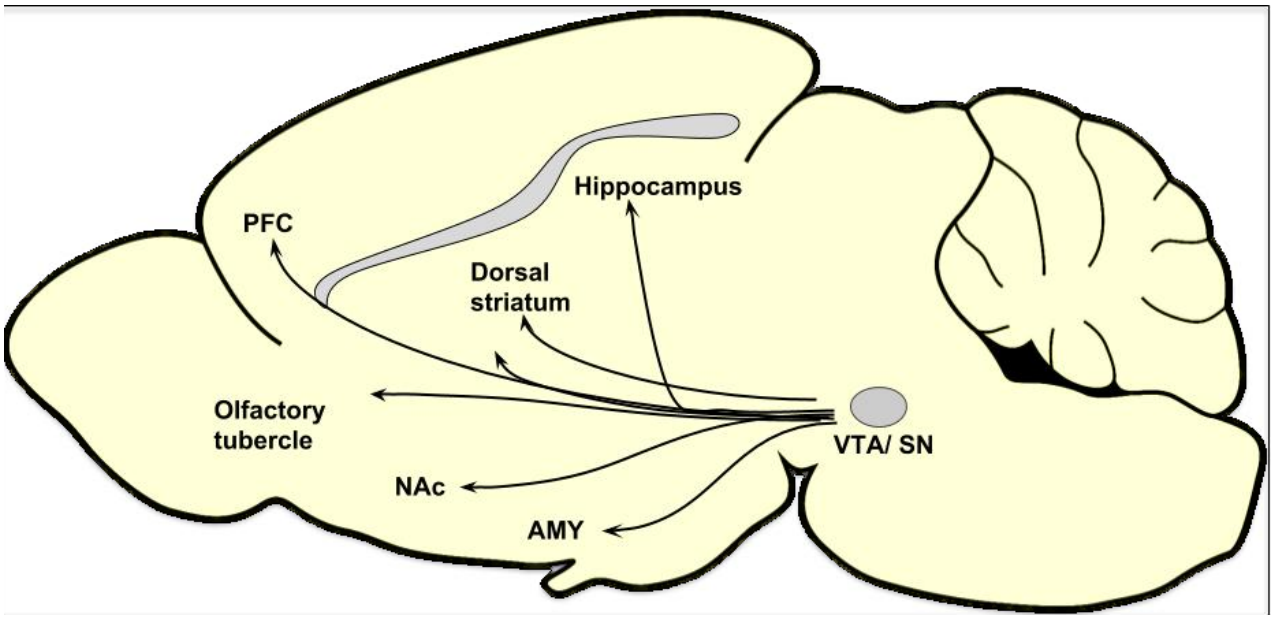


Figure 2 Reward pathway: dopaminergic neurons
Mesocorticolimbic (neurons from the VTA) and nigrostriatal (neurons from SN) systems. AMY: amygdala; NAc: nucleus accumbens; PFC: prefrontal cortex; SN: substantia nigra; VTA: ventral tegmental area.

In general, all substance of abuse activates either directly or indirectly the brain rewarding system (Di Chiara, 2000; Koob and Nestler, 1997; Nutt, 1996; Rowell et al., 1987). Psychostimulants, such as cocaine and amphetamine, activate the pathway by inhibiting the endogenous

monoamines (DA, serotonin, noradrenaline) reuptake from the synapses or by increasing its release from presynaptic neurons through interfering with monoamine transporter proteins (Hall et al., 2004; Uhl et al., 2002). This results in a flood of DA, serotonin (5-HT) and noradrenaline (NA) in the synapse, producing the stimulant effects of cocaine (Fig. 3). Nevertheless, it is believed that the psychostimulant effects, e.g. euphoria, are mainly caused by DA (Nestler, 2005).

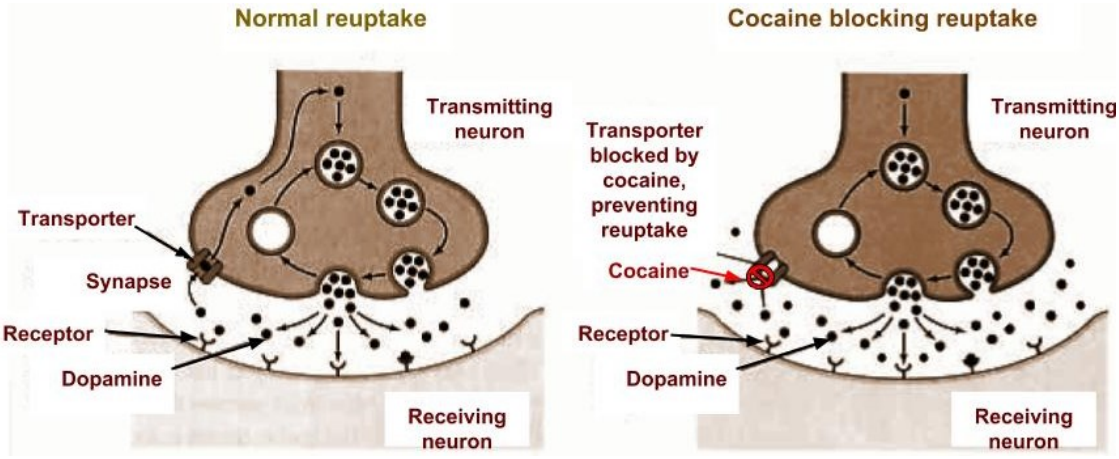


Figure 3 Cocaine action on the transporters
Cocaine inhibits the endogenous monoamines (dopamine, serotonin, noradrenaline) reuptake from the synapses. Adapted from Abadinsky (2004).

In the last decades, DA's role has been extended beyond the reinforcing effects of natural or drug rewards. It has been shown that DA affects motivation and attention to salient stimuli. For instance, lesions of dopaminergic neurons or infusions of DA receptor antagonists in the NAc reduced motivation (*wanting*) without affecting the hedonic value (*liking*) (Berridge and Robinson, 1998; Robinson and Berridge, 2001). This gains more relevance in the clinic as addicted patients often report their desire for drugs (wanting) continues despite the reduced pleasure (liking), caused by pharmacological tolerance. These studies demonstrate that not only *wanting* and *liking* can be dissociated, but also that dopamine is involved in other phenomena important for drug addiction (Nestler, 2005).

1.3 Definition of drug addiction

Drug addiction or severe SUD is a mental illness associated with dysfunctions at emotional, behavioral and cognitive scales. It can be defined as a chronic relapsing disorder characterized by a continued drug use in greater quantities and more frequently than intended despite the desire to abstain and the negative consequences, such as financial problems and poor health conditions (APA, 2018). Addicted individuals frequently feel an intense urge and desire to use the drug, usually accompanied by physical and/or psychological symptoms.

Two international classification of diseases are available to help medical doctors in the diagnosis, the International Statistical Classification of Diseases and Related Health Problems 10th revision (ICD-10), issued by the World Health Organization (WHO, 2016), and The Diagnostic and Statistical Manual of Mental Disorders (DSM-5), released by the American Psychiatric Associations (APA, 2013). ICD-11 was recently revised and its beta-draft version launched on June 18, 2018. However, ICD-11 will be officially out in May 2019 and clinically implemented by 2022. Therefore, ICD-10 will be commented in this thesis as it is the current classification used by the medical doctors. ICD-10 defines a drug abuser as addicted if he/she is simultaneously positive for three of the six criteria during the previous year. DSM released some changes in the last revision in 2013, where *drug addiction or dependence* is no longer used as definition of addiction, instead the severities of SUD is graded according to the number of criteria met. DSM provides a list of eleven criteria, and the diagnosis is as follow: 0–1, unaffected; 2–3, mild; 4–5, moderate; ≥6, severe.

Table 1 Criteria for drug addiction diagnosis

ICD-10	DSM-5
Criteria for substance dependence in ICD-10: I. A strong desire or sense of compulsion to take the substance; II. Difficulties in controlling substance-taking behavior in terms of its onset, termination, or levels of use;	I. Recurrent failure to fulfill major role obligations II. Recurrent substance use in physically hazardous situations III. Continued substance use despite persistent or recurrent social or

III. A physiological withdrawal state when substance use has ceased or been reduced, as evidenced by: the characteristic withdrawal syndrome for the substance; or use of the same (or a closely related) substance with the intention of relieving or avoiding withdrawal symptoms; IV. Evidence of tolerance, such that increased doses of the psychoactive substance are required in order to achieve effects originally produced by lower doses; V. Progressive neglect of alternative pleasures or interests because of psychoactive substance use, increased amount of time necessary to obtain or take the substance or to recover from its effects; VI. Persisting with substance use despite clear evidence of overtly harmful consequences, such as harm to the liver through excessive drinking, depressive mood states consequent to heavy substance use, or drug-related impairment of cognitive functioning. Efforts should be made to determine that the user was actually, or could be expected to be, aware of the nature and extent of the harm.	interpersonal problems IV. Tolerance V. Withdrawal VI. The substance is often taken in larger amounts or over a longer period than intended VII. Persistent desire or unsuccessful efforts to cut down VIII. Craving IX. Considerable time spent in obtaining the substance or using, or recovering from its effects X. Important social, work, or recreational activities given up because of use XI. Continued use despite knowledge of problems caused by or aggravated by use

Both ICD-10 and DSM-5 criteria are comparable, as shown in the Table 1 above. In this thesis, the focus will be on DSM because the animal model of cocaine addiction used in this thesis is based on the DSM. The 5th revision of DSM published in 2013 had some changes. Compared to

the DSM-IV, the criterion for craving was introduced and the criterion of recurrent legal problems was excluded (Piazza and Deroche-Gamonet, 2013). Craving is in fact an important feature of addiction because it leads to compulsive drug seeking and taking, culminating in relapse even after a long period of withdrawal. Drug paraphernalia, places and partners of drug use can promptly trigger drug craving and cause recurrent relapses. This last is one of the major challenges to overcome in drug addiction treatments. Although it is not yet clear what changes on the brain lead to the loss of control over the drug seeking and taking, several addiction theories have attempted to explain this abnormal behavior. Each theory highlights different aspects of the pathology. Below the main theories of addiction and the drug seeking behavior phenomena will be introduced.

1.4 Drug-seeking behavior and recurrent relapses

Relapses to self-administration can be reactivated following a period of withdrawal, and this phenomenon is found in both human and animals. The memory retrieval of drug-related experiences can cause anticipation of the pleasurable experience, producing craving (Childress et al., 1993; Childress et al., 1986; Ehrman et al., 1991; O'Brien et al., 1990; Robbins et al., 1997). Craving and drug-seeking behavior can eventually lead to reinstatement of the self-administration (Robbins and Everitt, 1999). Three main events appear to be involved in relapse. The first is taking the drug itself; the second is the conditioned stimulus, and in rats or mouse it can be the cue-light or the noise predicting drug availability (Berridge and Robinson, 1998; de Wit and Stewart, 1981; Di Chiara et al., 1999; Ito et al., 2000; Koob and Le Moal, 2001; Markou et al., 1993); and the third is inducing a state of stress, which in preclinical research foot-shocks are often used. All the three triggers have been shown to lead to similar neural events that are enough to activate drug-seeking behavior (Robbins and Everitt, 1999). In the present thesis, the focus was on the cue-elicited drug seeking behavior because it models key aspects of relapse in human addicts and reveals the involvement of limbic cortical–ventral-striatal systems in cocaine addiction (Everitt and Wolf, 2002).

Among the neuronal systems that play a role in conditioning and reinstatement of cocaine self-administration, glutamatergic and dopaminergic transmissions are certainly involved. Basal

extracellular glutamate levels are found reduced in the NAc before presentation of cocaine-associated cues, while cue-presentation or cocaine priming produced elevation of glutamate levels in this region (Hotsenpiller et al., 2001; McFarland et al., 2003). Intra-accumbal injections of glutamate receptor agonists can induce cocaine reinstatement (Cornish et al., 1999; Cornish and Kalivas, 2000). Particularly, alphaamino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/ kainate and N-methyl-D-aspartate (NMDA) receptors antagonists are able to attenuate reinstatement of cocaine seeking induced by cocaine priming or cocaine-associated cues (Backstrom and Hyytia, 2007; Cornish and Kalivas, 2000; Park et al., 2002). Cocaine cues also increases DA release in the NAc (Di Ciano et al., 1998; Ito et al., 2000) and appears to modulate drug reinforcement. Administration of DA receptor agonists can potentiate conditioned reinforcement (Taylor and Robbins, 1986; Weissenborn et al., 1996; Wolterink et al., 1993) and reinstate drug self-administration (de Wit and Stewart, 1981, 1983; Self et al., 1996; Shaham et al., 1994; Stewart, 1983; Stewart and Vezina, 1988), whereas antagonists of DA or glutamatergic AMPA receptors in the NAc core, but not shell, greatly attenuates cocaine-priming or cue-controlled reinstatements (Di Ciano and Everitt, 2001) (Bell et al., 2000; Bell and Kalivas, 1996; Kaddis et al., 1995; Layer et al., 1993). Other brain regions have also been implicated in drug-seeking behavior. Inactivation or lesions of basolateral amygdala (BLA) prevented cue-controlled cocaine seeking (Grimm and See, 2000; Kantak et al., 2002; Meil and See, 1997; Whitelaw et al., 1996), whereas inactivation of the central amygdala (CeA) prevented the acquisition of associative learning with cocaine-paired cues (Kruzich and See, 2001). Although antagonism of AMPA or NMDA receptors in the BLA failed to prevent cocaine cue-induced reinstatement (Di Ciano and Everitt, 2001; See et al., 2001), infusion of a D1, but not a D2, DA receptor antagonist did prevent it (See et al., 2001). The mPFC-striatum connections also appear important for the reinstatement of cocaine seeking (McFarland and Kalivas, 2001). Rats with lesions of the anterior cingulate cortex (ACC) or medial PFC (mPFC) showed persistent responding for cocaine, which was no longer under the control of contingent presentations of cocaine-related stimuli (Weissenborn et al., 1996). Furthermore, antagonism of DA receptor in the mPFC, but not in the striatum, abolished the relapse-inducing effects of cocaine (McFarland and Kalivas, 2001). Taken these studies together, activation of the PFC, AMY, and NAc are suggested to induce drug seeking in laboratory animals (Cornish and Kalivas, 2000; Hayes et al., 2003; McFarland and

Kalivas, 2001), whereas inactivation of these regions appears to attenuate cocaine seeking triggered by drug-associated cues (Fuchs et al., 2005; Grimm and See, 2000; McLaughlin and See, 2003). Involvement of these brain regions is consistent with human functional imaging studies during craving studies in abstinent drug users (Childress et al., 1999; Grant et al., 1996; Kilts et al., 2001; Schneider et al., 2001). Other limbic areas greatly activated during craving triggered by drug-related cues are striatum and OFC (Childress et al., 1999; Daglish and Nutt, 2003; Daglish et al., 2001; Garavan et al., 2000; Grant et al., 1996; Maas et al., 1998; Volkow et al., 2006; Wang et al., 1999). OFC, in particular, plays a role in reward valuation and integrates emotion and behavior (Bechara et al., 2000; Levy and Glimcher, 2012). Psychostimulant abusers often show impairments of OFC-related functions, such as poor judgement, maladaptive decision-making and a rigid habit system, which produces deficits in flexible and adaptive behavior (Lucantonio et al., 2012). Therefore, these corticolimbic structures appear to greatly contribute to mechanisms controlling cocaine seeking.

1.5 Main addiction theories

Several theories of drug addiction have attempted to explain the abnormal behaviors displayed but drug addicted subjects. Below, I will discuss the most relevant theories.

1.5.1 Incentive salience

DA system has been heavily studied in the past decades because of its role in reward, and the incentive salience theory is very much based on dopaminergic phenomena (Deadwyler, 2010; Everitt, 1990; Hernandez and Hoebel, 1988; Schott et al., 2008; Wise and Rompre, 1989). The incentive motivation or incentive-sensitization theory was introduced by Robinson and Berridge (2008) and hypothesizes that repeated administration of addictive drugs produce long-lasting changes in the brain organization systems that regulate the reward and motivation processes, causing hypersensitivity to drugs and drug-associated stimuli (Robinson and Berridge, 2001). The enhanced DA signaling in the mesocorticolimbic region caused by the use of addictive drugs hypersensitizes the DA system and increases the incentive salience of events correlated with drug-taking, affecting the decision making. In other words, the hypersensitization state changes

the perception of drug-related stimuli with salience, enhancing its value and making them attractive and "wanted". These neuroadaptations elicit approach to the drug and also craving, making them susceptible to relapse after discontinuation of drug use (Berridge, 2007, 2012). The researchers also suggest that 'wanting' and 'liking' for hedonic rewards can be separated, where 'wanting' rather than hedonic 'liking' is dependent on DA system. Consequently, sensitization to the drug related stimuli can continue provoking "wanting" despite the development of tolerance and the reduced subjective pleasurable effects of the drug (Berridge et al., 2009; Robinson and Berridge, 1993, 2008). Several studies indeed support this theory. For instance, behavioral sensitization to amphetamine in rats show increased DA firing patterns in striatum, a brain region that code for the incentive salience of reward-conditioned stimuli (Tindell et al., 2005). Similarly, PET studies in humans have demonstrated that repeated amphetamine administration increases DA release with successive doses (Boileau et al., 2006), and drug-related stimuli produce robust DA responses in the brain reward systems (Boileau et al., 2007; Childress et al., 2008). These results support the theory's fundamentals that drug-induced sensitization in brain DA systems in drug addiction mediates Pavlovian conditioned incentive motivational processes (i.e., attributing salience), thereby invigorating drug-related motivation (i.e., wanting) and drug-reinforced responding (Berridge and Robinson, 2016; Bickel et al., 2018).

1.5.2 Deficits in prefrontal cortical function

It has been postulated that the loss of control over drug-seeking and taking behavior is a combination of three components: (1) drug-induced enhancements of stimulus-reward learning, (2) increased behavioral control by reward-related stimuli and (3) impairments of frontostriatal systems involved in inhibitory control (Jentsch et al., 1999; Robbins and Everitt, 1999; Shallice and Burgess, 1996). It has been proposed that drug-induced dysfunction of the prefrontal cortex, amygdala and striatum, which are cortical and subcortical brain regions involved in motivation, learning, memory and cognition, lead to simultaneous reduced capacity for response inhibition and increased behavioral control by reward-related stimuli (Bechara et al., 2000; Everitt et al., 2008; Volkow and Fowler, 2000). In fact, impairments of the frontal cortical regions are suggested to prevent the suppression of rapid conditioned responses/reflexes and preclude guidance of behavior by slower cognitive mechanisms, evidences shown by many other

pathological states, including schizophrenia and obsessive compulsive disorder (Fey, 1951; Robbins, 1990, 1996; Damasio, 1996).

Neuroimaging studies in psychostimulant abusers have indeed reported hypofunction of prefrontal cortical areas and related cognitive dysfunction (Goldstein and Volkow, 2011), supporting this theory. Particularly, the OFC has been found dysfunctional in cocaine addiction, which was correlated with loss of inhibitory control mechanisms (Jentsch et al., 2002; Volkow and Fowler, 2000). Curiously, a FDG-PET study found OFC hyperactive during acute drug withdrawal of active cocaine abusers (Volkow et al., 1991), whereas hypoactive during protracted abstinence (Adinoff et al., 2012). Increased OFC activation is also found in obsessive-compulsive disorders (Insel, 1992), and therefore it is postulated that drug-associated stimuli reactivate OFC in addicted subjects during withdrawal, contributing to compulsive drug-seeking and taking behaviors (Volkow et al., 2003). Other neuroimaging studies assessing craving reported activation of other brain structures recruited in executive functions (Everitt et al., 2001), including ACC, AMY, striatum and dorsolateral PFC (dlPFC) (Childress et al., 1999; Grant et al., 1996; Maas et al., 1998; Wang et al., 1999). Preclinical data are consistent with these findings. OFC has been shown to distinguish rewarding and punishing outcomes, necessary to recognize prediction errors (Liu et al., 2007; O'Doherty, 2007), and lesions of this brain region result in behavioral compulsion to obtain the reward despite the lack of its reinforcing properties (Rolls, 2000). Other studies in monkeys have demonstrated that lesions of dlPFC, lateral orbitofrontal cortex or the ventromedial frontal cortex result in augmented perseveration and inhibitory deficits (Butter, 1964; Butter et al., 1970; Diamond and Goldman-Rakic, 1989; Dias et al., 1996; Goldman et al., 1971; Iversen and Mishkin, 1970; Moll and Kuypers, 1977; Ridley et al., 1993; Rolls, 1996; Rosenkilde, 1979). Akin to these findings, monkeys that chronically self-administer amphetamine showed impairments of frontal cortical cognitive function, attention deficits, verbal memory and delayed recall as well as “behavioral disinhibition” (Castner and Goldman-Rakic, 1999; McKetin and Mattick, 1998; Rogers et al., 1999). Within the ventromedial PFC, the prelimbic region (Prl) appears to be particularly important in drug addiction, given its position within the limbic circuitry. It provides direct inputs to the NAc and is interconnected with AMY (Barbas, 1993; Krettek and Price, 1974; Morecraft et al., 1992; Nauta, 1962). Lesions of Prl

impair extinction of an operant response in monkeys (Baylis and Gaffan, 1991; Butter, 1964) and are suggested to produce deficits in the ability to adapt the behavior in face of changes in the contingencies. Taken these studies together, dysfunctional frontal lobe in drug addiction appears to cause the inability to inhibit the behavioral response towards the originally reinforced stimulus that has been contingently associated with primary reward (Jentsch et al., 1999).

1.5.3 Aberrant learning and habit formation

Aberrant learning and habit formation theories also consider the role of DA in learning and memory processes in addiction, in particular the striatum and the PFC. In the late 1980s, Norman White began to suggest addiction is a disorder of learning and mnemonic processes, as studies started to link striatum with associative learning and memory processes (White, 1989, 1996). In the following years, the relevance of associative learning for human addiction further gained attention as drug taking, including relapses after periods of abstinence, follows exposure to cues previously associated with drug use (Childress et al., 1993; O'Brien et al., 1998; Tiffany, 1990; Wikler and Pescor, 1967). Based on research with natural rewards, Anthony Dickinson then developed the formal associative learning hypothesis (Dickinson et al., 1996). The researchers showed that during the experimentation phase of natural rewards, the subject is driven by a goal-directed behavior in order to acquire the desired reward or object (Balleine and Dickinson, 1998; Dickinson et al., 1996). Following repeated training, decision processes are transferred from goal-directed to become more habitual (Dickinson et al., 2002). This idea was further decoded into behavioral research with addictive drugs initiated by Trevor Robbins and Barry Everitt (Everitt et al., 2001; Everitt and Robbins, 2005).

The learning and habit theories hypothesize that drug addiction is the endpoint of a series of alterations from initial voluntary drug use to the loss of control over drug-seeking and –taking (Bickel et al., 2018). The chronic drug use pathologically disrupt or usurp the learning and memory systems due to the large increase in DA levels produced by the drug, leading to the establishment of compulsive seeking habits that occurs at the expense of other reinforcement sources (Everitt et al., 2001; Everitt and Robbins, 2005, 2016; Hyman et al., 2006). Therefore, the initial drug-seeking that is under the control of goal-directed processes becomes compulsive

under the control of habit processes with the onset of addiction. Although habit mechanism can be quite helpful to capture any successful behavior in order to rapidly rescue subjects from dangerous situations, this mechanism also makes them (or us) more vulnerable to maladaptive behaviors, such as in the case of drug addiction (Everitt and Robbins, 2016). According to these theories, the change from a voluntary and goal-directed drug use to more habitual and compulsive drug use depend on the neuroplasticity and transitions from PFC and ventral striatum to the control of dorsal striatum (Everitt et al., 2008; Everitt and Wolf, 2002; Hyman et al., 2006). In fact, plasticity of neural system in psychostimulant addiction has shown gradual strengthening and involvement of dorsal striatum (DS) following chronic drug self-administration (Everitt and Wolf, 2002). During early periods of drug-seeking, *in vivo* microdialysis studies have found increased DA release in the NAc core. However, DA release was elevated in the DS during prolonged periods of drug-seeking (Ito et al., 2000; Ito et al., 2002). DA release in this brain region also appears to produce maintenance of drug seeking, whereas blockade of these receptor significantly reduce cocaine seeking (Vanderschuren et al., 2005). Downregulation of striatal DA D2 receptors is also a consistent marker of chronic cocaine (Goldstein and Volkow, 2002) and alcohol (Heinz et al., 2004) abuse in humans. Hence, the learning and habit formation theories of addiction postulate that abusive drugs promote strong learning of 'automatized' stimulus–response (S–R) habits, and that their nature S–R habits confer compulsivity to behavior (Berke and Hyman, 2000; Everitt et al., 2001; Hyman et al., 2006; Tiffany, 1990). These two processes can be studied by separating Pavlovian and instrumental learning processes. Pavlovian learning is sensitive to the contingency between stimuli and outcomes, whereas instrumental processes are sensitive to the contingencies between actions or responses and outcomes (Bickel et al., 2018).

1.5.4 Opponent process

The opponent process theory proposes that addiction is a cycle of spiraling dysregulation of positive and negative reinforcement, where three stages are constantly repeated: preoccupation/ anticipation, binge/ intoxication, and withdrawal/ negative affect (Koob, 2008; Koob and Le Moal, 2001; Koob and Nestler, 1997). It was first proposed by Solomon and Corbit (1974) and then extended by Koob and Le Moal (2001). The earliest opponent process theory stated that the positive hedonic response occurs during the initial acute drug effects, but is subsequently followed by an opposed process that dampens this positive response, such as the development of

tolerance. Therefore, the initial hedonic process occurs rapidly after the drug use and, as the initial process ends, a homeostatic response to oppose these initial effects begins with slow onset and decay, increasing in its magnitude after repeated exposure. Chronic drug users indeed report reduced hedonic effects of the drug (tolerance) and increased aversive states during withdrawal.

Koob and Le Moal (1997) describe an allostatic model of brain motivational systems to explain the behavioral changes of addicted patients as the simple homeostatic imbalance appears insufficient. Allostasis is a process of achieving a homeostatic state (stability) from the deviation of the brain regulatory systems produced by the chronic drug abuse. The accumulated damage to the brain systems over time results in neuroadaptive changes, creating conditions that support relapse even after protracted abstinence (Bickel et al., 2018). Therefore, the opponent process theory postulates that addiction is a cycle of increasingly dysregulated brain reward and anti-reward systems that drives to compulsive drug use; in other words, the drug is no longer used to benefit from its pleasurable effect, but to alleviate the withdrawal symptoms (Koob and Le Moal, 2001; Solomon, 1980). The brain reward system includes the activation of networks in the VTA and NAc, which mediate the hedonic as well as aversive effects, whereas the anti-reward system includes neuroadaptations within-system and/ or between-systems. Within-system adaptations refer to molecular or cellular changes within a singular reward system, producing reduced positive hedonic effects of the drug with an overlay of negative hedonic valence (e.g. mesolimbic DA and opioid function decreases during withdrawal after chronic drug use) (Koob, 2008). Between-system adaptation is the changes in other circuitry when one single brain system is excessively recruited and activated. For example, brain stress and emotional circuits are recruited after repeated activation of the brain reward system, providing an additional source of negative hedonic valence. Hence, as withdrawal and dependence develop, corticotropin-releasing factor (CRF) and noradrenergic neurons are recruited producing aversive states marked by irritability, anhedonia, dysphoria and loss of motivation of unconditional rewards (Koob, 2003; Nestler, 2001).

1.6 Dysregulation of circadian rhythm

Although dysregulation of circadian rhythm is not a diagnostic criterion in DSM-IV/5 or ICD11, drug abusers often show disturbed sleep-wake cycle as well as reduced levels of melatonin and delayed nocturnal peak of melatonin (Ford and Kamerow, 1989; Vengeliene et al., 2015).

Circadian rhythm of most of mammals' physiological processes, such as body temperature, sleep-wakefulness, hormone release and metabolism, are controlled by suprachiasmatic nucleus (SCN) (Buijs and Kalsbeek, 2001) and synchronized by the light/dark cycle (Klein and Moore, 1979). During the dark phase, melatonin (N-acetyl-5-methoxytryptamine) is secreted by pineal gland, while during the day its synthesis is suppressed by light (Hardeland et al., 2012; Lynch et al., 1975b). Melatonin produces hypnogenic (Sugden, 1983), muscle-relaxing (Kovacs et al., 1974), and anticonvulsant (Albertson et al., 1981) effects, likely by modulating dopaminergic, glutamatergic and GABAergic neurons (Cardinali, 1981; Castillo-Romero et al., 1993; Datta and King, 1980). Two melatonin receptors — MT_1 and MT_2 — have been characterize and both subtypes are coupled to G-proteins that mediate adenylyl cyclase inhibition. MT_1 receptors also activate phospholipase C beta and MT_2 additionally inhibits guanylyl cyclase pathway (von Gall et al., 2002).

Disturbed circadian rhythms are long known in aging and some psychiatric disorders, such as Alzheimer and major depression (Linkowski et al., 1987; Mirmiran et al., 1992). However, researchers have observed that drug sensitization, consumption, and preference vary depending on the time-of-day of drug administration, suggesting that melatonin levels may be involved in drug reinforcing effects (Akhisaroglu et al., 2004; Brick et al., 1984; Garmabi et al., 2016; Hasler et al., 2012; Kurtuncu et al., 2004). In fact, moderate suppression of melatonin levels is found after acute alcohol intake as well as delayed melatonin peaks have been demonstrated in both chronic alcohol and cocaine abusers or chronically-treated rats (Ekman et al., 1993; Hasler et al., 2012; Prosser et al., 2014; Rojdmark et al., 1993; Vengeliene et al., 2015). Alcohol and other substance of abuse also appear to entrain the body clock (Shibata et al. 2010), and this disruption in the circadian rhythm are suggested to be involved in drug addiction (Abarca et al., 2002; Conroy et al., 2012; Kovanen et al., 2010; McClung, 2007; McClung et al., 2005; Spanagel et al.,

2005b). In addition, alterations in protein expression levels of circadian clock genes, including *Clock*, *Per 1* and *Per 2,* have been linked to substance abuse (Akhisaroglu et al., 2004; Brick et al., 1984; Garmabi et al., 2016; Kurtuncu et al., 2004; Manev and Uz, 2006; Prosser et al., 2014; Uz et al., 2005a; Yuferov et al., 2003). These genes can regulate dopaminergic transmission, and thus play a role in drugs of abuse-induced effects (McClung et al., 2005). Melatonin receptors are also found in brain regions involved in hedonic drug effects as well as in the maintenance of drug use, such as PFC, NAc, DS, AMY and hippocampus (Noori et al., 2012; Uz et al., 2005b). Therefore, manipulating circadian system, such as melatonergic system, may influence addictive behaviors and be potentially a complementary medication for SUD treatments. In fact, Vengeliene et al (2015) have demonstrated abolishment of relapse-like ethanol consumption in rats following administration of melatonin or a non-selective melatonin receptor agonist agomelatine.

1.7 Neural alterations in psychostimulant dependence

The sporadic drug use to compulsive behavior involves neuroplasticity in several neuronal circuits that process not only motivation and reward, but also memory and habits, executive function and inhibitory control (Koob and Volkow, 2010). These neuroadaptations produce changes in the levels and dynamics of several neurotransmitter systems, including glutamate and gamma-aminobutyric acid (GABA), in the limbic and frontal cortical circuitry, as previously described. Within this circuitry, activation of AMY, NAc, DS, and PFC have been shown essential for drug addiction (Fig. 4). Below, the role and neuronal projections of those brain regions in drug addiction will be introduced.

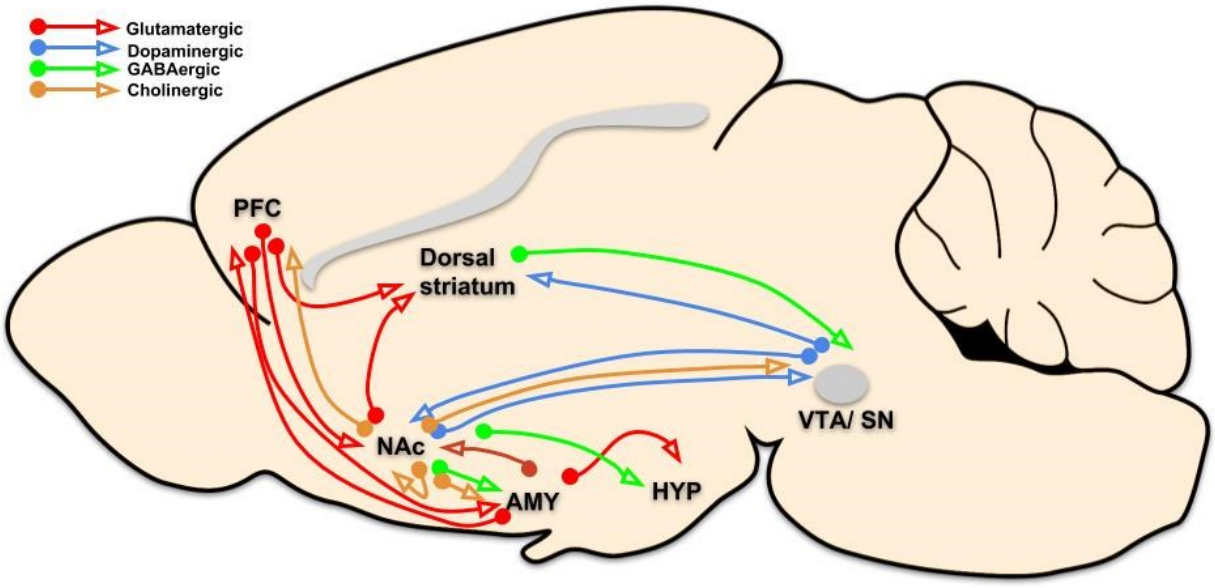


Figure 4 Simplified schematic of connectivity between regions playing a role in addiction
Neuronal projections within brain regions that play a role in psychostimulant addiction. AMY: amygdala; HYP: hypothalamus; NAc: nucleus accumbens; PFC: prefrontal cortex; VTA/SN: ventral tegmental area/ substantia nigra.

Amygdala consists of a heterogeneous group of interconnected nuclei that is critical for learned associations between motivationally relevant events and the neutral stimuli that predict fear or reward (Kalivas and Volkow, 2005; Wolf, 2016). This learning involves recruitment of neurons that segregate according to the valence, which means neurons responding to reward cues do not respond to fear cues (Janak and Tye, 2015). BLA and CeA show some functional dissociations, although they work together to process some aspect of reward (Wolf, 2016). The glutamatergic projections from BLA to NAc is necessary for motivated behavior driven by reward-associated cues (Everitt and Robbins, 2005; Everitt and Wolf, 2002; Feltenstein and See, 2013). CeA is a major output nucleus for amygdala projections to hypothalamus and brain stem, in particular the CRF transmission within the CeA that plays a role in the drug withdrawal state (Koob, 2014).

Nucleus accumbens consists of two functionally and structurally distinct compartments, the shell and the core, and the majority of its neurons (90-95 %) are GABAergic projections known as medium spiny neurons (MSNs). The MSNs receive convergent glutamate inputs from limbic and

cortical regions that are important for regulating and generating motivated behaviors, playing a crucial role in translating motivation into action (Mogenson et al., 1980; Sesack and Grace, 2010). In return, those neurons project to motor-related regions that enable the execution of these behaviors (Meredith et al., 2008; Sesack and Grace, 2010). The shell is strongly interconnected with ventral tegmental area and hypothalamus, being important for appetitive behaviors (Kelley, 2004; Robinson and Berridge, 1993). Reciprocal dopamine projections from the VTA to the NAc shell modulate motivational salience and participate in establishing learned associations between motivational events and environmental stimuli (Bassareo and Di Chiara, 1999; Cheng, 2003; Ito et al., 2000; Sellings and Clarke, 2003). The core compartment is anatomically associated with the orbitofrontal and the anterior cingulate cortex, and is suggested to mediate the expression of learned behaviors in response to cues predicting motivationally relevant events (Di Ciano and Everitt, 2001; Kelley, 2004). Furthermore, the involvement of the accumbens core in the expression of the adaptive behavior depends not on dopaminergic afferents, but on glutamatergic afferents from the prefrontal cortex (Di Ciano and Everitt, 2001).

The *dorsal striatum* receives a series of convergent excitatory projection inputs from prefrontal cortical regions, insula, motor and sensory cortex, thalamic nuclei, hippocampus, and amygdala, which are under dopaminergic and serotonergic neuromodulatory control (Gerfen, 1984; Kelley and Domesick, 1982; Malach and Graybiel, 1986; Pan et al., 2010; Voorn et al., 2004). These excitatory projections diffusely synapse onto MSNs expressing D_1 or D_2 dopamine receptors in the dorsal striatum, in which D_1R MSNs project via direct pathway to the substantia nigra pars reticulate/internal segment of the globus pallidus (GPi or entopeduncular nucleus in rodents) and D_2R MSNs project via indirect pathway to the external segment of the globus palliudus (GPe) (Gerfen, 1984; Gerfen and Surmeier, 2011; Kreitzer and Malenka, 2008; Surmeier et al., 1993). Dorsal striatum is involved in action initiation, learning, context- and cue-induced reinstatement of cocaine (Bossert et al., 2013; Feltenstein and See, 2013) as well as in goal-directed and habit formation (Everitt and Robbins, 2016). The goal-directed systems in rodents and humans rely on interactions between the posterior dorsomedial striatum and the mPFC (Everitt and Robbins, 2016; Shiflett et al., 2010; Yin et al., 2006). Conversely, the habit system depends on the anterior dorsolateral striatum, or putamen in humans, and the motor cortex (Balleine and O'Doherty,

2010; Yin et al., 2004). For both goal-directed and habits processes, dopamine modulates the interactions between cortical afferents and striatal processes (Everitt and Wolf, 2002). Although increase of DA levels in the NAc is particularly observed in initial drug effects (Di Chiara and Imperato, 1988), following chronic drug self-administration the DA transmission is increased in both NAc and DS regions (Everitt and Wolf, 2002; Letchworth et al., 2001; Nestler, 2001).

Prefrontal cortex is crucial for behavioral flexibility, inhibitory response control, working memory and decision making processes. The rodent ventral-medial PFC can be subdivided into anterior cingulate, prelimbic, infralimbic, and orbitofrontal cortices (Dalley et al., 2004; Heidbreder and Groenewegen, 2003). PFC reciprocally communicates via excitatory glutamatergic projections to and from amygdala as well as sends excitatory outputs to the NAc (Kalivas, 2007). It also receives inputs from basal ganglia, substantia nigra, ventral tegmental area, lateral hypothalamus and hippocampus as well as thalamocortical projections that project to different areas of the PFC (Dalley et al., 2004). The prelimbic region particularly appears to be more reactive to drug-associated cues that may drive in part by enhanced glutamate release in the mPFC during drug seeking behavior (Shin et al., 2016; Wolf, 2016). OFC is recruited during motivationally relevant events and plays a role in distinguishing stimuli that predict such events, contributing to the behavioral response (Breiter et al., 2001; Bush et al., 2002). Nevertheless, because PFC targets several neurotransmitter systems, including noradrenaline-containing neurons, dopamine, serotonin and acetylcholine neurons, PFC functions as neuromodulatory cortical network influencing other cortical processes and excitatory synaptic transmission (Dalley et al., 2004).

It has been demonstrated that chronic cocaine use induce short- and long-term alterations in the firings of DA neurons in the VTA (Bonci et al., 2003; Koob and Volkow, 2010) as well as alterations in the synaptic plasticity. Long-term potentiation (LTP) of AMPA-mediated excitatory neurotransmission in DA neurons are blocked after psychostimulant expoure (Kauer, 2004; Saal et al., 2003; Thomas and Malenka, 2003; Ungless et al., 2001). Cocaine self-administration also impairs long-term depression (LTD) in the nucleus accumbens and PFC (Kasanetz et al., 2010; Kasanetz et al., 2013). Recent studies have also identified persistent cellular changes in glutamate

transmission that may be critical for the neuroadaptations to psychostimulant use. A marked change reported was the increase density of dendritic spines in the NAc in postsynaptic glutamatergic transmission (Robinson and Kolb, 2004), which appears accompanied by an increase in the insertion of AMPA receptors into the membrane of spiny neurons in that region (Boudreau and Wolf, 2005; Kalivas, 2007). Other proteins regulating the postsynaptic glutamate transmission were also changed after chronic cocaine use, including postsynaptic density (PSD)-95 and Homer proteins (Swanson et al., 2001; Yao et al., 2004). This psychostimulant-induced postsynaptic neuroplasticity appears associated with alteration in the biochemical machinery regulating the spine formation, particularly increasing in actin cycling and formation of F-actin, a structural protein that regulates the spine morphology and the insertion of proteins in and out of the membrane (Toda et al., 2010). These studies indicate that synaptically released glutamate will be interpreted by postsynaptic cells (Kalivas, 2007). Nevertheless, it is remarkable that the adaptation in glutamate transmission that promotes presynaptic glutamate release in response to a stimulus could arise from adaptations that decrease inhibitory presynaptic regulation by metabotropic glutamate inhibitory autoreceptors (mGluR2/3) (Baker et al., 2003; Bowers and Kalivas, 2003). Reduction in basal extracellular levels of glutamate in the NAc after withdrawal from cocaine has also been reported and it is thought to result from reduced glial cystine-glutamate exchange (Baker et al., 2003). The cystine-glutamate exchanger is the rate-limiting step in the synthesis of glutathione and controls the majority of extracellular glutamate levels outside the synaptic cleft (Baker et al., 2002). Activation of this exchanger by pro-cysteine drugs blocks the cocaine reinstatement by stimulating inhibitory presynaptic mGluR2/3 and restoring levels of extrasynaptic glutamate (Baker et al., 2003; Moran et al., 2005). Deletion of the Homer2 gene in the NAc showed remarkable similarities to animals withdrawn from chronic treatment with cocaine, including increased behavioral responsiveness to cocaine as well as reduced cysteine-glutamate exchange and increased probability for glutamate release (Szumlinski et al., 2004). PSD-95 gene deletion also increased the acute behavioral response to cocaine (Yao et al., 2004).

1.8 0/3crit animal model of cocaine addiction

Several factors, e.g. familiar background, early onset of drug use, and environmental conditions, interact during the course of drug use, contributing to the severity of the disease. Nevertheless, individuals who do not show any of the known risk factors to addiction can still develop the pathology, suggesting that divergent functional or molecular changes on the brain may define the development of addiction or resilience to addiction. Therefore, understanding the adaptations of neural networks from the initial drug exposure and throughout the chronic use is fundamental to the progress of successful clinical treatments, which is currently deficient for psychostimulant dependence. Animal models are useful for that due to their more controlled experimental conditions than human studies as well as for enabling to perform longitudinal studies. In this thesis, the 0/3crit model of cocaine addiction was used.

The 0/3crit model of cocaine addiction in rats was developed by Deroche-Gamonet et al. (2004). Different to a typical cocaine self-administration (CSA) experiments that lasts 10 ~ 30 days, the 0/3crit model of cocaine addiction consists of at least 45 CSA sessions (~ 2.5 months) prior to the behavioral assessments for addiction-like behaviors. The CSA session is explained in the Fig. 5 below.

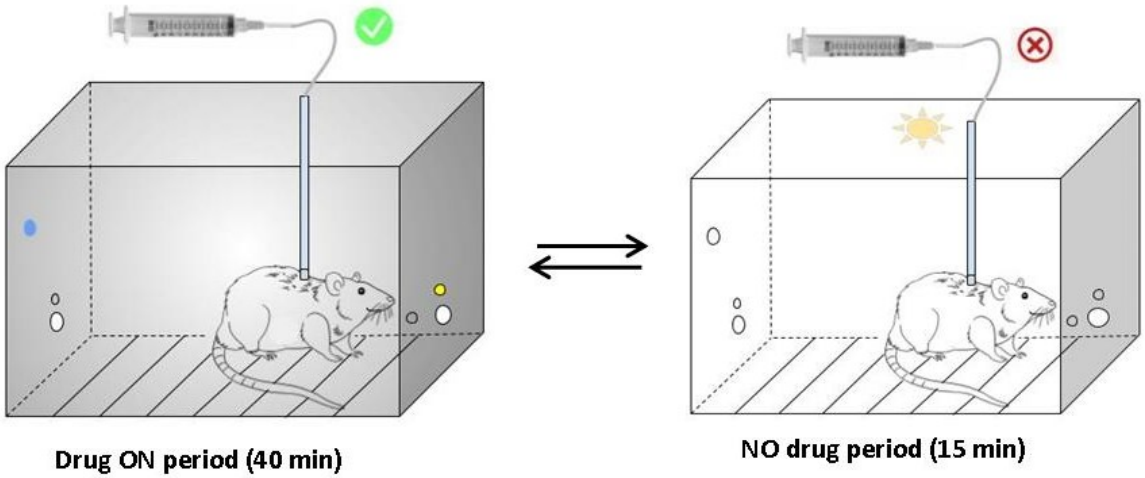
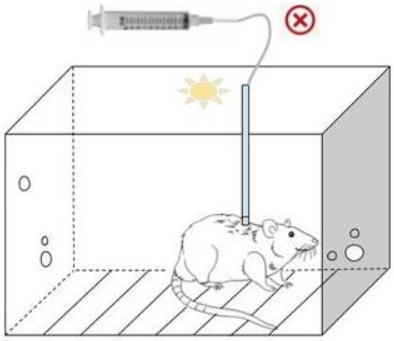


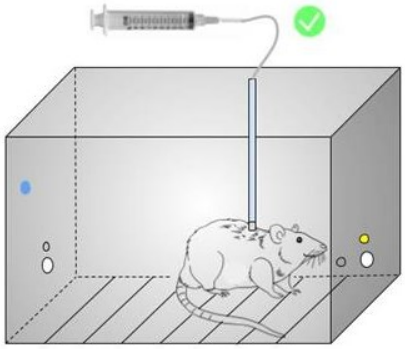
Figure 5 Cocaine self-administration training
Cocaine self-administration (CSA) consisted of alternated periods of drug availability (Drug ON) and unavailability (NO drug). During the Drug ON period, the blue cue-light signaled the availability of cocaine and nose-pokes were scheduled to fixed ratio 5, i.e. five nose-pokes in the active hole to deliver one cocaine infusion (0.8 mg/kg). Every cocaine infusion was paired with a white cue-light right above the active hole. During the NO drug period, a house light indicated the unavailability of cocaine. Cue-lights and cocaine infusion were withdrawn, but nose-pokes were recorded. A CSA session lasted a total of 2h30, which consisted of three-Drug ON and two-NO drug periods.

Following the long-term CSA training, the three main DSM-IV/5-based behavioral criteria for substance use disorder are then assessed in the animals, as shown in the Fig. 6.



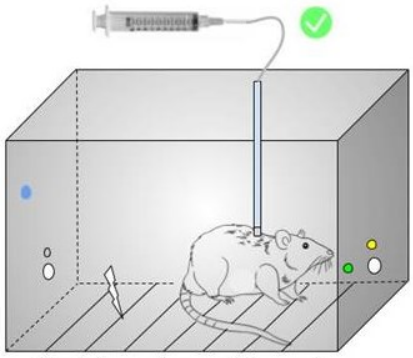
Persistence to seek the drug

(1) ***Subjects struggle stopping drug use or limiting drug intake.*** This is assessed by the persistence to nose-poke, calculated by the sum of active nose-pokes during the NO-drug periods between CSA sessions 40–44. Addicted-like rats increase the number of nose-pokes during this period after repeated CSA training.



Break Point test (up to 6h)

(2) ***Subjects have enormously high motivation to take the drug, with activities focused on its procurement and consumption.*** This is assessed by the break point test (BP) in a progressive-ratio schedule on CSA 45. For each cocaine infusion, animals are required to respond progressively higher within the CSA session. The maximal score received before the cessation of responding is considered to be a reliable index of their motivation. The BP is as follow: 10, 20, 30, 45, 65, 85, 115, 145, 185, 225, 275, 325, 385, 445, 515, 585, 665, 745, 835, 925, 1025, 1125, 1235, 1345, 1465, 1585.



Resistance to punishment

(3) ***Subjects maintain the substance use despite the harmful consequences.*** This is assessed by the persistence to take cocaine regardless of punishment, i.e. a mild foot-shock (0.2 mA) paired with cocaine infusion. It is performed on CSA 50 and expressed as the percentage of infusions received in this test in relation to the baseline infusions (averaged between CSA sessions 47-49).

Figure 6 Assessments of addiction-like behaviors

The DSM-based behavioral criteria for addiction are tested in rats subjected to at least 45 CSA sessions: Persistence to seek the drug when drug is not available, high motivation to take the drug and persistence of self-administration despite negative consequences (i.e., the application of an electric foot-shock).

For each of the above addiction criteria, a score of either 0 or 1 is given to each rat according to their performance during the assessments. Rats showing responses above the 60th percentile of the population distribution in a defined test received a score of 1, whereas all below the chosen cutoff received a score of 0. Summing the scores of all three assessments, each rat will exhibit total scores between 0 and 3. Animals that met all 3 criteria are classified as addicted-like (3crit), whereas those with none or zero, as non-addicted-like (0crit) rats. These two distinct groups of animals are of special interest because despite their similarity in the genetic background, environmental conditions as well as in the number of cocaine infusions throughout the training, their motivation and performance towards cocaine seeking is strikingly different. Furthermore, the fraction of animals that display addiction-like behaviors (15 - 18%) is comparable to human cocaine addicts (Anthony et al., 1994; Nutt, 1996).

1.9 Aims

In order to uncover some of the differences in the brain activity of compulsive and controlled drug users, the 3crit model of cocaine addiction was explored in this thesis. The aim of this doctoral thesis was to combine different research techniques to identify vulnerability and protective hallmarks on the brains of cocaine addicted-like (3crit) and non-addicted-like rats (0crit).

Specific aims:

- Carry out a longitudinal multimodal magnetic resonance imaging study to identify functional and structural changes on brains of 0crit and 3crit rats;
- Assess brain function of 0crit and 3crit rats by Positron Emission Tomography (PET);
- Map immediate early gene expression of Arc in several brain regions following drug-induced reinstatement test in 0crit and 3crit groups;
- Evaluate the dendritic spine density in the medial PFC;
- Test novel drugs as potential medications for treatment of cocaine addiction;
- Evaluate differences in the motivational influences of the conditioned stimuli between 0crit and 3crit groups.

Chapter 2: Neuroimaging

Whole-brain *in vivo* neuroimaging has brought rapid and profound impact to investigate brain function in high-resolution anatomy and functional connections. Not surprising, the discovery of resonance phenomenon used in the magnetic resonance imaging (MRI) received four Nobel Prizes; the first Nobel Prize was in Physics, awarded in 1952 to Felix Bloch and Edward Mills Purcell for the development of precise measurements of nuclear resonance in different materials' compositions, followed by two Nobel Prizes in Chemistry, in 1991 to Richard Ernst for his contributions to the development of high resolution nuclear magnetic resonance spectroscopy, and in 2002 to Kurt Wüthrich for the development of the three-dimensional nuclear magnetic resonance spectroscopy to measure biological macromolecules structure in solution. The last, but not least, Nobel Prize in Physiology or Medicine was awarded to Paul C Lauterbur and Peter Mansfield in 2003 for the development of magnetic resonance into a useful imaging method to visualize different structures. Development of other imaging techniques, such as computer-assisted tomography also received a Nobel Prize in Physiology or Medicine in 1979, awarded to Allan M Cormack and Godfrey Newbold Hounsfield (Prize, 2018). All these discoveries have led to a breakthrough advance in medical diagnostics and research by allowing harmless examination of almost all organs in the body. Today neuroimaging machines are available for both humans and small animals. However, only few longitudinal studies have been performed in preclinical research to date. Despite scarce literature, those few studies have shed light to our understanding of the brain function and structure. For instance, an elegant longitudinal MRI study performed in monkeys found a direct relationship between voluntary ethanol intake and decrease in brain gray matter volume (Kroenke et al., 2014). The researchers also showed that the pattern of volumetric changes can be observed following 15 months of alcohol drinking and that greater volume shrinkage occurred in monkeys that had early onset of alcohol drinking and that became heavier drinkers in adulthood (Kroenke et al., 2014). Other longitudinal studies have demonstrated that both rodent (Sullivan et al., 2006) and human brains (Bartzokis et al., 2002; Bartzokis et al., 2004) grow until late adulthood, changing our basic understanding about brain development. To our knowledge, longitudinal MRI study in cocaine addiction has not been performed to date.

Hence in this thesis, the 0/3crit translational animal model of cocaine addiction was used to investigate vulnerability and protective markers for cocaine addiction and resilience.

2.1 Magnetic Resonance Imaging

MRI became the gold standard for radiology by providing high resolution images with good contrast between different tissues. The essence of the MRI is the nuclear magnetic resonance (NMR) spectroscopy. NMR uses the absorption and emission of radiofrequency electromagnetic radiation (RF) by the nuclei placed in a magnetic field. In short, the nucleus of a Hydrogen atom is a proton, which has a positive electrical charge and has a property called spin. A spinning proton will create an electrical current that is accompanied by a magnetic field. Therefore, it can be seen as a little bar magnet or compass needle. When the protons are placed in an external magnetic field, like the MRI scanner, it will align itself in parallel or anti-parallel direction to the magnetic field. Short bursts of radio frequency pulses can then be sent in to the protons, which will pick up energy and jump to a higher energy level. When the RF is switched off, the protons go back to their lower energy level, emitting the radiative absorption energy that will be detected as a MR signal. Using a gradient coil to slightly modulate the applied field, it is possible to measure small volume within the scanner. These small volumes are called voxels, providing a three-dimensional unit of resolution.

MRI can provide structural and functional images as above mentioned. Structural MRI uses the small differences between voxels to accommodate clinical discrimination of the tissues. Functional MRI (fMRI) requires speed of acquisition, normally at the expense of resolution. It measures brain anatomy as well as brain activity in an indirect manner. An activated area of the brain produces dynamic changes in blood flow and oxygen consumption. The oxygenated and deoxygenated hemoglobin show different magnetic properties, and produce small effects in the MR signal (Leniger-Follert and Lubbers, 1976). This effect is the basis of blood oxygenation level dependent (BOLD) contrast. Studies combining electrophysiology and fMRI have shown correlation between the increase in fMRI signal and spiking activity of neurons, demonstrating that fMRI is sensitive to changes in neural activity and thus, in brain function (Attwell and Iadecola, 2002; Logothetis, 2002, 2003; Logothetis and Wandell, 2004; Silva et al., 2000).

Neuroimaging studies in stimulant dependents have been inconsistent. Some of the structural MRI studies have shown enlargement of the basal ganglia and abnormal decrease in grey matter [GM] volume in several frontal areas, including anterior cingulate, insular, and orbitofrontal cortex, as well as reduction in temporoparietal and cerebellar cortex (Chang et al., 2005a; Ersche et al., 2011; Franklin et al., 2002; Jan et al., 2012; Jernigan et al., 2005). Conversely, other studies have demonstrated a decrease of the basal ganglia volume (Barros-Loscertales et al., 2011; Hanlon et al., 2011; Moreno-Lopez et al., 2012a) and reduced temporal GM volume (Bartzokis et al., 2000). Other neuroimaging studies have reported none structural abnormalities on brains of cocaine-addicts (Narayana et al., 2010; Weller et al., 2011).

fMRI studies have reported more consistent results than structural MRI. In human fMRI combined with successful no-go inhibitions have demonstrated impairment of inhibitory control in cocaine addicted subjects, which correlated with hypoactivity of anterior cingulate cortex and medial frontal areas (Kaufman et al., 2003; Li and Sinha, 2008). Right inferior frontal cortex has been reported to mediate response inhibition (Rubia et al., 2001; Rubia et al., 2003; Rubia et al., 2005), suggesting that cocaine addiction is accompanied by an impairment of neural connectivity critical for cognitive control (Li and Sinha, 2008). Activation of sensory association cortex, motor cortex, and posterior cingulate cortex were found increased during exposure to cocaine-related cues, indicating that those brain regions may be involved in relapse to cocaine abuse (Kosten et al., 2006). Cingulate cortex and OFC regions have also been found activated during exposure to conditioned cues that predict reward, and to elicit craving state (Koob and Volkow, 2010; McClernon et al., 2009). In another study investigating stress-induced cocaine craving, researchers reported increased activation of caudate and dorsal striatum regions in dependent subjects (Volkow et al., 2006). Taken these studies together, the frontal cortex appears dysfunctional in cocaine addicted subjects, which was correlated with impaired inhibitory control and craving.

Diffusion MRI or diffusion tensor imaging (DTI) studies also showed inconsistency in white matter integrity. DTI provides quantitative information about the relative direction of water

diffusion within a voxel, permitting to reconstruct the white matter tracts, known as tractography. There are four most common DTI parameters to measure the white matter integrity, the fractional anisotropy (FA), the mean diffusivity (MD), the longitudinal diffusivity (LD), and the radial diffusivity (RD). The concept of DTI is that restricted water diffusion perpendicular to the orientation of the axons can contribute to increase efficiency of neural conduction. Therefore, a high index of anisotropy is an indication of healthy and mature white matter microstructure. Nevertheless, there is no one-to-one relationship between a particular DTI parameter and a given anatomical feature. DTI studies in cocaine abusers have found lower FA in the inferior frontal white matter compared to controls, which was found correlated with length of cocaine use (Lim and Helpern, 2002; Lim et al., 2008). Moeller et al. (2005) have reported lower FA in the corpus callosum and increased RD, which is suggestive of alteration in white matter myelin, in cocaine dependent subjects compared with control subjects. The same group later reported lower FA in the splenium and higher RD in the posterior corpus callosum in cocaine dependent patients relative to controls (Ma et al., 2009).

The above mentioned studies are inconclusive and this may be caused by pre-existing features in the function or structure on brains of cocaine addicted subjects and/or the discrepancies may be resulted from environmental, physiological, pharmacological and genetic interactions (Gould et al., 2012), which certainly play a role in addiction vulnerability, maintenance, abstinence, and relapse. Based on this, longitudinal studies, particularly in animal models, are very helpful to clarify these issues due to their more controlled experimental designs, enabling to characterize within-subject neurobiological changes that are associated with long-term drug use. Identifying structural and functional changes from a naïve to a pathological state could shine light to our understanding of the development of the disease. Hence, a longitudinal study was performed on the 0/3crit translational animal model of cocaine addiction to investigate pre-existing differences and changes on brains of cocaine addicted-like and non-addicted-like rats.

Material and Methods

Animals

Male Sprague-Dawley rats weighing 250-300g at the beginning of the experiments were obtained from Charles River, Germany. The animals were kept in a 12 h reverse dark/light cycle (on 18h00, off 6h00), with temperature (23 ± 1°C) and humidity (50 ± 10 %) controlled. Standard laboratory rat food (Ssniff, Soest, Germany) was given 20g daily to avoid excessive weight gain due to small compartment for placing the animal in the MRI scanner. Water was provided *ad libitum* throughout the experimental period. All procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe, Germany) and were carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs

Cocaine hydrochloride (Sigma Aldrich, Taufkirchen, Germany) was dissolved in sterile saline.
Anesthetics: Isofluran (Baxter Deutschland GmbH, Unterschleissheim, Germany) and medetomidine (Domitor, Janssen-Cilag, Neuss).

Catheter implantation

A polyurethane catheter (internal diameter: 0.58 mm, external diameter: 0.94 mm) was implanted at the jugular vein under isoflurane anesthesia (~2%). The proximal end was placed in the right atrium of the animal's heart, while the distal end was passed underneath the skin and fixed in the mid scapular region. Rats were given 4-6 days of recovery before the first baseline MRI scan. Catheters were flushed daily with a heparinized solution (100 IU/ml) containing 1 mg/ml of enrofloxacin (Baytril®).

Operant cocaine self-administration apparatus

CSA was carried out in operant chambers (Imetronic, France) enclosed in ventilated sound-attenuating cubicles. Two nose-poke holes were located at the opposite walls, 5 cm above the grid floor. Nose-poke responses were recorded by the interruption of a photo-beam projected across the hole. Nose-pokes in the active hole resulted in a delivery of cocaine infusion, whereas nose-pokes in the inactive hole had no programmed consequences. The chambers were equipped

with a white cue-light located above the active nose-poke hole, a green cue-light next to it, a blue cue-light located on the opposite wall 33 cm above the grid floor and the house light that illuminated the entire chamber. Data was collected using POLY software.

Cocaine self-administration conditioning

CSA was based on Deroche-Gamonet et al. (2004) and Cannella et al. (2013). Briefly, each CSA session was comprised of drug availability periods (drug-ON, 40 min) alternated with NO-drug periods (15 min). During drug ON periods, blue cue light was lit to indicate the availability of cocaine and a white cue-light was paired with cocaine infusion. The fixed ratio for nose-poke in the active hole was 5 (FR5), i.e. 5 nose-pokes are required for a delivery of 0.8 mg/kg/infusion of cocaine. Each cocaine infusion was followed by a 40s time-out period. During NO-drug periods, cue-lights were withdrawn and the house light signaled the non-availability of cocaine. Nose-pokes had no scheduled consequences during NO-drug period. Each CSA session consisted of 2h30, divided in three drug-ON and two NO-drug periods, or a maximum of 35 cocaine infusions.

Following 44 CSA sessions, animals were tested for the three addiction criteria: (1) motivation for taking cocaine, (2) persistence of drug-seeking and (3) resistance to punishment.

Motivation for cocaine was assessed with a break point (BP) test. Blue and white cue lights were lit during the test and the progressive ratio of reinforcement was schedule as follow: 10, 20, 30, 45, 65, 85, 115, 145, 185, 225, 275, 325, 385, 445, 515, 585, 665, 745, 835, 925, 1025, 1125, 1235, 1345, 1465, 1585. The test ceased either after 5h or uncompleted ratio in a 1h period. The last completed ratio performed by the rat was used as representative of their motivation for cocaine.

Persistence of drug-seeking was analyzed as the mean active nose-pokes during NO-drug periods in the last three cocaine SA training prior to the BP test.

Resistance to punishment was assessed by pairing cocaine infusion and foot shocks (0.2 mA, 1s). In addition to the blue and white cue lights, a green cue-light was turned on at FR1 to indicate the presence of a shock. At FR4 animals received a foot shock, and at FR5 cocaine infusion was delivered paired with another foot shock. The test lasted 40 min and the criterion was expressed as percentage of cocaine infusions earned in relation to the baseline training in the first drug-ON period.

For the analysis of addiction-like behaviors, animals performing above 60th percentile of the population distribution for each of the criteria were considered positive and therefore classified as addicted-like (3crit) rats, whereas animals lower from that threshold were considered negative and therefore classified as non-addicted-like rats (0crit).

MRI acquisition

A total of four batches of animals (n= 58/ batch) were trained in the 0/3crit model to complete the group size for MRI study. The procedure in each batch of animals and MRI measurements was kept consistent. Three MRI scanning were performed within batch: (I) after 7 and 9 days from catheter implantation and prior to the CSA training; (II) between CSA sessions 14 – 16, and (III) between CSA sessions 54 and 56.

Experiments were carried out in a 9.4 Tesla MRI scanner (Bruker BioSpec, Ettlingen, Germany) with Avance III hardware, BGA12S gradient system (maximum strength 705 mT/m) and Paravision 6 software, using the linear whole-body volume transmitter coil combined with an anatomically shaped four-channel receive-only coil array for transmission and reception. Different anesthetic regimes were used for the different imaging sequences: 4% isoflurane in a mixture of N2 (70%) and O2 (30%) were initially used to induce the anesthetic state and thereafter reduced to 2.5% for maintenance. 30 minutes before beginning of the functional study, medetomidine was injected as a bolus (0.5 ml, 0.07 mg/kg, s.c.) and isoflurane progressively reduced (0.5% every 2 min) to zero. Medetomidine was continually infused (0.28 mg/kg/h) until the end of the experiment. Breathing and cardiac signals were recorded (10-ms resolution) during the measurements using the signal breakout module (Small Animal Instruments Inc., NY, USA) and the 4-channel recorder (Velleman® N.V., Gavere, Belgium). Ten minutes after starting the rs-fMRI sequence, a cocaine (or saline) bolus was injected (0.9 mg/kg) at the rate of 1.2 ml/min.

The MRI acquisition protocol included a DTI sequence and structural image.

DTI data were acquired using an Echo Planar Imaging spin-echo diffusion sequence with the following parameters: TR 5000 ms, TE 19 ms, 35 gradient orientations with b-value 1000 s/mm^2 plus five non-diffusion weighted images, matrix size = 128 x 128 x 16, in-plane resolution =

0.384 x 0.0384 mm^2, slice thickness = 0.5 mm (gap = 0.2 mm). Respiratory triggering was used in order to minimize motion artefacts.

Structural brain image was acquired using a T2-weighted rapid acquisition with refocused echoes (RARE) sequence with the following parameters: RARE factor 16, TR/TE 1200/50 ms, flip angle 180°, the voxel dimension 0.15 mm, acquisition time 23 min.
The total acquisition time of the sequences was 150 min per animal.

Data analysis

Behavioral characterization of addiction

The behavioral performance in the addiction-like tests was normalized within batch of animals to make comparable classification of addicted-like and non-addicted-like between experimental batches.
For comparison between 3crit and 0crit rats in each of addiction-like behavioral criteria, a student t-test analysis was used. The chosen level of significance was $P < 0.05$. Data were analyzed using GraphPad Prism software.

MRI scalars analysis

Individual longitudinal differences were calculated by subtracting the values to its own control (Baseline scanning): $\Delta = scan_{> 50\ CSA} - scan_{Baseline}$. Voxelwise comparisons were carried out in order to identify longitudinal group-specific cocaine-induced differences in structure (GM volume, volume, GM density) or water diffusivity (FA, MD, LD and RD). Voxelwise tests were limited to within-brain voxels (whole-brain analysis) or to the white matter skeleton (TBSS analysis). Statistical inferences of changes in MRI parameters were performed by General Linear Method (GLM) implemented in SPM12 package. The obtained statistical maps were corrected for multiple comparisons using Threshold Free Cluster Enhancement (TFCE) at the level of $P<0.05$ (FDR). In order to support voxel-wise findings ROI-based analysis were performed over 46 bilateral anatomical regions. Average MRI scalars were extracted across all voxels within region.
In order to investigate the relationship with inborn features anticipating further cocaine addiction-wise behaviors, maps and roi-wise MRI scalars derived from baseline scanning were correlated

with the three normalized behavioral measures reflecting motivation (BP test), resistance to punishment (% baseline infusions) and persistence (AP during non-drug periods) and the addiction score (AS). Statistical inferences in MRI parameters maps were performed by General Linear Method (GLM) implemented in SPM12 package. The obtained statistical maps were corrected for multiple comparisons using Threshold Free Cluster Enhancement (TFCE) at the level of $P<0.05$ (uncorrected). ROI-based correlations were calculated as the Spearman correlation over 46 anatomical regions. Correlations below $P<0.05$ (uncorrected) were considered as positive relationship.

Structural imaging preprocessing

Structural data was corrected for field inhomogeneity; brain extracted using 3D pulse-coupled neural networks (Chou et al., 2011). Subsequently, preprocessed data were segmented in gray mater (GM), white matter (Erickson et al.) and cerebrospinal fluid (CSF) tissues by in-house generated tissue probability maps (TPM). In short, preprocessed data were segmented using FAST v.4.0 (FMRIB's Automated Segmentation Tool) and affinely transformed to the standard Paxinos and Watson space (Paxinos and Watson, 2007). Study-specific compartments templates were averaged across subjects. Identified compartments were classified as GM, CSF and WM/CSF. WM/CSF compartment was additionally segmented in three new compartments (2 corresponding to WM and 1 correspondig to CSF). GM, WM and CSF tissue probability maps were generated by adding subtypes compartments.

Eventually structural data were segmented using the within-study TPM and normalized by SPM12 (SPM12, http://www.fil.ion.ucl.ac.uk/spm/software/spm12). To account for local expansion and shrinking of the brain, GM segments were modulated with the determinant of the transformation Jacobian. Normalized GM segments, normalized and modulated GM segments and determinant of the transformation Jacobian were smoothed (FWHM = 0.6 mm × 0.6 mm × 0.6 mm).

DTI Preprocessing

Eddy current and motion distortions were corrected by affine registration. Tensor estimation was done with the software Camino (Cook et al., 2006). The tensor model was fitted using a Robust Estimation of Tensors by Outlier Rejection approach (Chang et al., 2005b), after which the

following parameter maps were computed for each data set: fractional anisotropy (FA), mean diffusivity (MD), axial diffusivity (AD), radial diffusivity (RD). Estimated FA maps were spatially normalized to study-specific FA template aligned with anatomical template. Computed deformation field was used to spatially normalize MD, LD and RD maps.

In order to evaluate white matter alterations, FA maps were fed into an in-house modified version of the Tract-Based Spatial Statistics (TBSS) routine of FSL19. TBSS produces an accurate normalization of white matter tracts in comparison to whole-brain voxel-wise analyses. After skeleton extraction, skeletonized maps were obtained for all parameters (FA, MD, RD, LD) by applying the pre-computed registration and skeletonization steps.

Results

The neuroimaging data was entirely analyzed by Dr. Alejandro Cosa-Linan.

Characterization of addiction-like behaviors

Behavioral characterization of non-addicted like (0crit) and addicted-like (3crit) rats was performed after 44 CSA sessions. The persistence of cocaine-seeking was calculated by the sum of active nose-pokes during the unavailability periods between CSA sessions 40–44. Motivation for cocaine intake was represented by the last completed progressive ratio in the break point test on CSA session 45. Resistance to punishment was expressed as percentage of cocaine infusions paired with foot shock earned in comparison to baseline infusions, tested on CSA session 50. Student t-test revealed significant difference between 0crit and 3crit rats in each addiction-like criterion [Fig. 7; persistence of cocaine seeking: $t(28)=5.24$; motivation to take the drug: $t(28)=10.36$; resistance to punishment: $t(28)=5.74$; $P<0.0001$].

A total of four batches of animals were performed to complete the group size for the longitudinal MRI study. Among them, eleven 3crit and nineteen 0crits were scanned in the MRI.

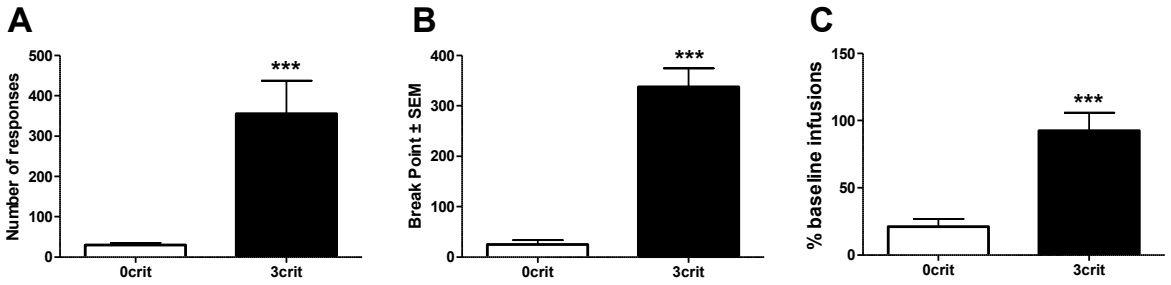


Figure 7 Characterization of addiction-like behaviors
Behavioral characterization of non-addicted like (0crit) and addicted-like (3crit) rats for the longitudinal MRI study. (A) Persistence of cocaine-seeking calculated, (B) motivation for cocaine intake, and (C) resistance to punishment. Student t-test revealed significant difference between 0crit (n=19) and 3crit (n = 11) rats in each addiction-like criterion. Data are shown as mean ± SEM; ***P < 0.0001

Addicted-like rats show increased grey matter volume

The grey matter volume of addicted-like rats (3crit) was found significantly increased in several brain areas of the limbic structures compared to 0crit and control groups. The comparison was made between the first and the third scanning time-points, and it indicates that addiction pathology produce morphological changes in the brain compared to both control and non-addicted rats. Specifically, 0crit rats showed GM increase in the caudate putamen (corrected, CPu, $P<0.05$) and entohirnal cortex (Ent, $P<0.01$) compared to control and 3crit rats, while 3crit showed GM increase in olfactory tubercle (tu, $P<0.01$), prelimbic cortex (PL, $P<0.01$), cingulate cortex 1 (Cg1, $P<0.01$), ventral and globus pallidum (VP and GP, $P<0.05$ and $P<0.01$, respectively), nucleus accumbens (Acb, $P<0.01$), secondary motor cortex (M2, $P<0.01$), and substantia nigra (SN, $P<0.01$) compared to control or 0crit groups (Fig. 8). Furthermore, control and 0crit rats showed similar GM volumes in all other brain regions not mentioned above, indicating that 0crit brains may be able to maintain morphology caused by cocaine or the pathology.

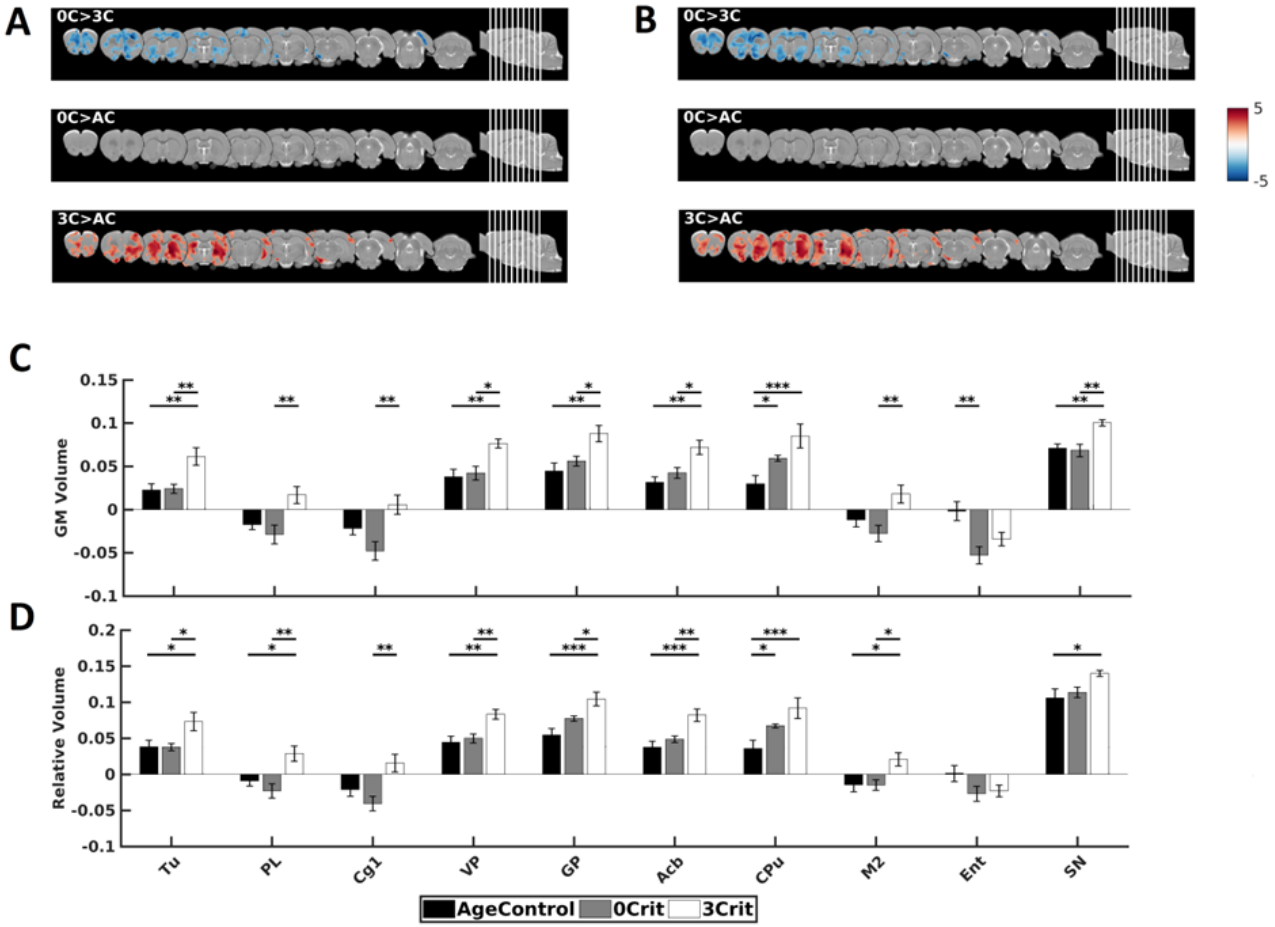


Figure 8 Structural changes in 0/3Crit rats between the first and the last MRI scan
A) Statistical parametric maps of the pairwise GM volume changes for voxel-wise analysis. (B) Statistical parametric maps of the pairwise volume (assessed by Jacobian determinants) differences for the voxel-wise analysis. Additionally, ROI-based comparison of GM volume (C) and relative volume (D) were performed. Only regions surviving FDR correction are shown. Maps were thresholded at the level of P<0.05 (FDR corrected). *, **, *** indicates P<0.05, P<0.01, P<0.001, respectively. 0C: 0crit, 3C: 3crit, AC: Age Control.

The morphological changes on the brain can be observed following short-term cocaine exposure in VP, GP, Tu, CPu, Acb and SN, as the Fig.9 shows the relative GM changes in all three scanning time-points (corrected P<0.05).

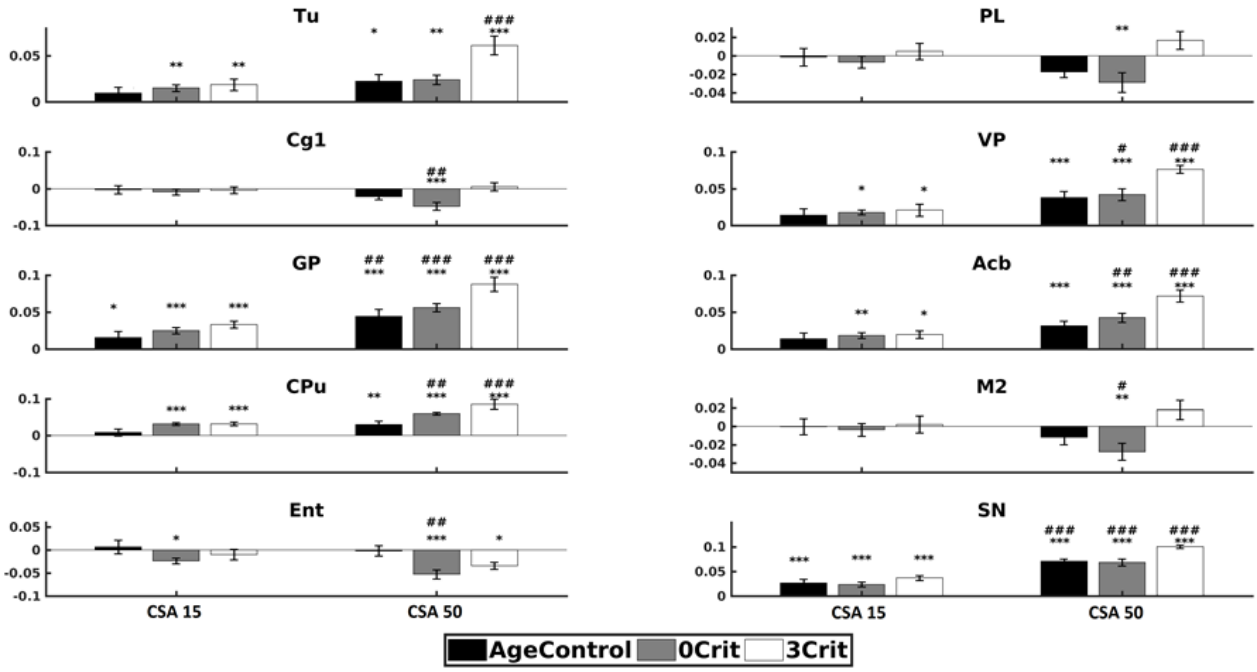


Figure 9 ROI-based GM volume in 0/3Crit rats relative to first scan
GM volume changes in ~CSA15 and ~CSA50 in relation to first MRI scanning in several brain regions. Maps were thresholded at the level of P<0.05 (FDR corrected) and only regions surviving FDR correction are shown. Tu (olfactory tubercle), PL (prelimbic cortex), Cg1 (cingulate cortex 1), VP (ventral pallidum), GP (globus pallidum), Acb (nucleus accumbens), CPu (caudate putamen), M2 (secondary motor cortex), Ent (entorhinal cortex), and SN (substantia nigra). *, **, *** and t* indicates P<0.05, P<0.01, P<0.001 and P<0.1 respectively compared to baseline; #, ##, ### and t# indicates P<0.05, P<0.01, P<0.001 and P<0.1 respectively compared to ~ 15 CSA.

Diffusion tensor imaging reveals changes in the non-addicted-like rats

Microstructural changes can produce alterations of DTI parameters. However, the exact relationship between them and the underlying tissue structure is still a matter of debate (Scholz et al., 2014). The DTI measurement compared the first and third MR scanning time-points (Fig. 10) and it reveals that in many brain regions the white matter structures were altered in 0crit in comparison to 3crit or control groups (corrected P<0.05). Among the brain structures that showed WM changes, the thalamus compartments (DLT, MDT, VMT), zona incerta (ZI), cingulate cortex 2 (Cg2) and SN showed increased MD, RD and/or LD in the 0crit compared to 3crit and/or control groups. The MD, RD and LD were decreased in frontal association cortex (FrA) and retrosplenial cortex (RS) in both 0crit and 3crit groups as compared to control group.

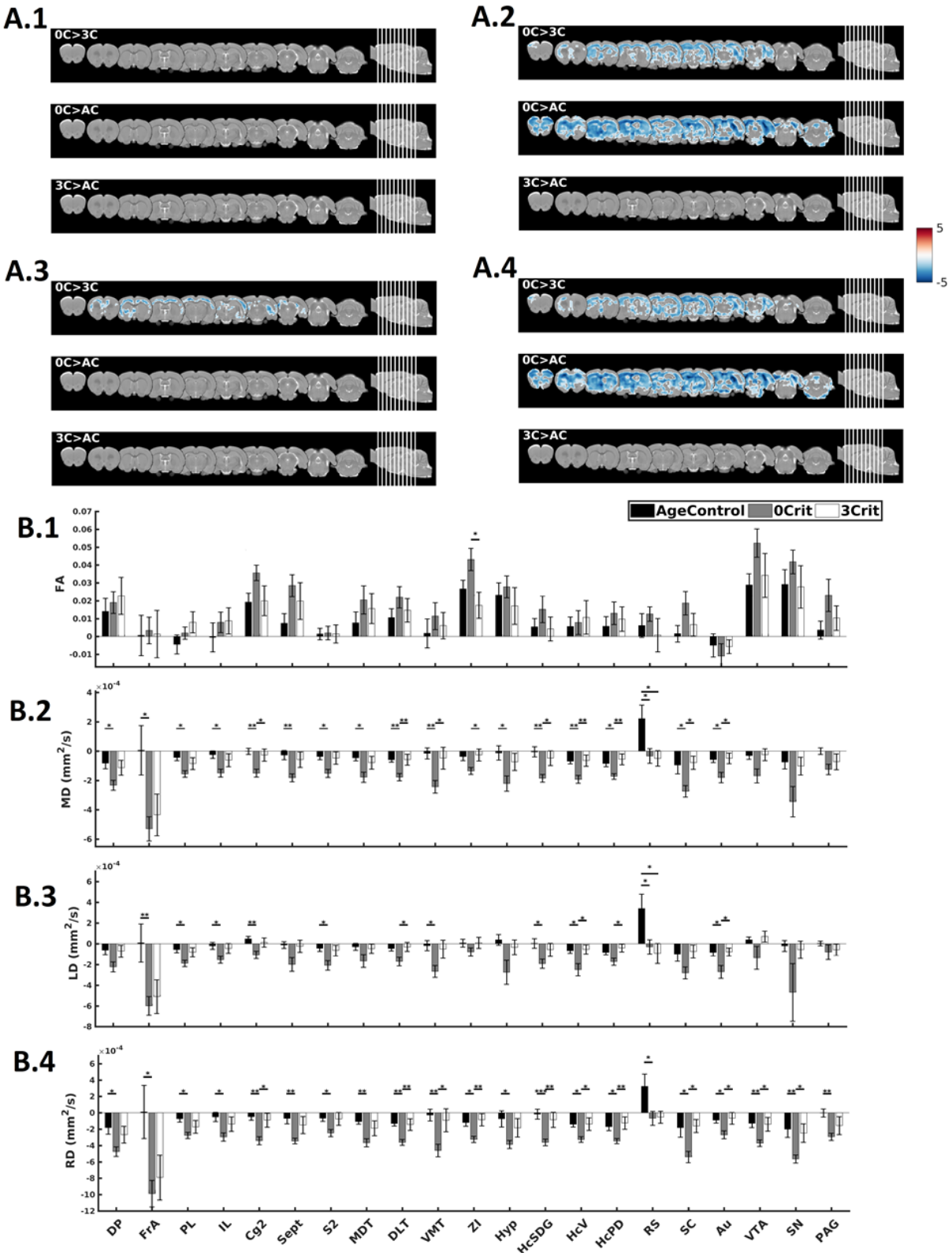


Figure 10 Changes in water diffusivity in 0/3Crit rats between the first and the last MRI scan

Statistical parametric maps of the pairwise changes for voxel-wise tests, FA (**A.1**), MD (**A.2**), LD (**A.3**), RD (**A.4**). Additionally, ROI-based comparison of FA (**B.1**), MD (**B.2**), LD (**B.3**), RD (**B.4**) were performed. Only regions surviving FDR correction are shown. Maps were thresholded at the level of $P<0.05$ (FDR corrected). *, **, *** indicates $P<0.05$, $P<0.01$, $P<0.001$, respectively. 0C: 0crit, 3C: 3crit, AC: Age Control.

The mean values of the white matter skeleton analysis in the three scanning time-points showed differences within group in comparison to the first measurement (baseline) (Fig. 11). However, the variation in the tissue characteristics appears to be not large enough for significant difference in the diffusion measures (uncorrected $P<0.05$).

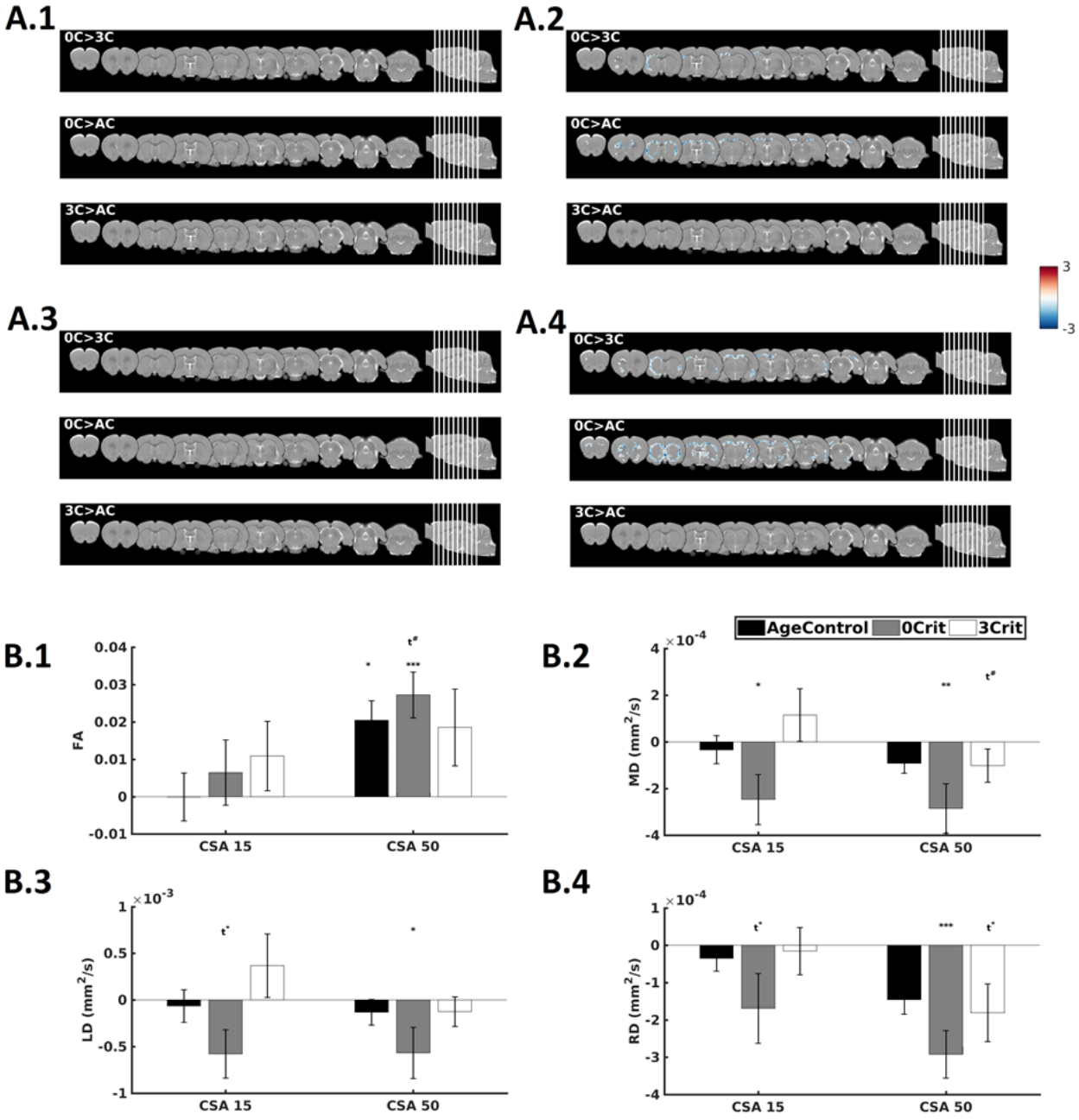


Figure 11 Changes in water diffusivity in the white matter skeleton of 0/3Crit rats in the second and third MRI measurements in relation to baseline

Statistical parametric maps of the pairwise changes for voxel-wise analysis, FA (A.1), MD (A.2), LD (A.3), RD (A.4). White matter skeleton mean values are shown in FA (B.1), MD (B.2), LD (B.3), RD (B.4). TBSS maps were thresholded at the level of P<0.05 (uncorrected). *, **, *** indicates P<0.05, P<0.01, P<0.001, respectively, in comparison to baseline. #, ##, ### and t# indicates P<0.05, P<0.01, P<0.001 and P<0.1 respectively, in comparison to ~ CSA 15. 0C: 0crit, 3C: 3crit, AC: Age Control.

Discussion

The present study demonstrates that along the course of cocaine use addicted-like rats showed increased structural (grey matter volume) changes in several brain regions compared to both 0crit and control rats. Changes were found in olfactory tubercle, prelimbic cortex, cingulate cortex, ventral and globus pallidum, nucleus accumbens, caudate putamen and substantia nigra. In some brain regions, changes were visible after ~15 cocaine self-administration sessions, although clear evidences are found after 55 CSA sessions. White matter integrity analysis revealed changes in different DTI parameters in several brain regions. Among them, the fractional anisotropy was altered in zona incerta in 0crit relative to 3crit. Mean group diffusivity indicates no significant difference between groups, however within group changes are seen over the course of drug use.

The grey matter volume (GM) analyzed with voxel-based morphometry (VBM) is a relative straightforward analysis because it quantifies the amount of GM in a voxel, permitting comparison across subjects (Winkler et al., 2010). By aligning the images to a standard brain, volumes are corrected for regional expansions and shrinkages using the Jacobian determinants (Good et al., 2001). Nevertheless, interpretation of GM volume data is still unclear because there is no direct relationship between GM volume and pathological substrate. GM changes have been documented in several psychiatric pathologies. For instance, GM atrophy was found in frontal and temporal cortices in schizophrenia patients (Gur et al., 1999; Hulshoff Pol et al., 2002; Nakamura et al., 2007), whereas increased GM volume was reported in autism spectrum disorder (Wang et al., 2017) and in individuals that practice physical activity (Erickson et al., 2010). Taking those results together, it appears that changes in the GM volume cannot indicate healthy or pathological states, considering that changes in the GM goes in different directions in different psychiatric pathologies. In psychostimulant dependents, studies have been inconsistent, reporting 'no changes' (Narayana et al., 2010; Weller et al., 2011), increase (Chang et al., 2005a; Ersche et al., 2011; Franklin et al., 2002; Jan et al., 2012; Jernigan et al., 2005) or decrease (Barros-Loscertales et al., 2011; Hanlon et al., 2011; Moreno-Lopez et al., 2012a) of GM volumes in different brain regions. These discrepancies in the human studies may be caused by pre-existing differences in the GM volume, either genetically differences in the brain structure or changes

produced by physiological variabilities, e.g. food intake (Roberto et al., 2011), steroids, (excess) body fat (Janowitz et al., 2015), medication such as mood stabilizer lithium (Moore et al., 2000; Sassi et al., 2002), tobacco smoking (Sutherland et al., 2016), and alcohol abuse (Thayer et al., 2016) can all affect the GM volume (Amiri et al., 2018). Therefore, in clinical studies it is impossible to dissociate changes caused by cocaine use from pre-existing variabilities at the moment of the study. In the present longitudinal study, in comparison to non-addicted-like and control groups, cocaine addicted-like rats showed increased GM volume in brain regions that participate in the rewarding effects of cocaine, including nucleus accumbens, olfactory tubercle, caudate putamen, prelimbic, and ventral and globus pallidum. Conversely, GM changes between non-addicted-like and control groups were akin over the course of drug use, except in the caudate putamen, indicating that 0crit brains showed normal age development. The dorsal striatum, which is part of caudate and putamen, is a brain region essential for habitual behavior (Everitt and Robbins, 2016); therefore, the repeated instrumental responses during the cocaine self-administration training may have caused alteration in GM of this brain region. Thus, it appears that 0crit may not be affected by chronic cocaine use along life, having therefore protecting mechanisms to keep the brain under normal functioning, and/or 3crit brains may be more sensitive to toxicity of cocaine.

In the longitudinal DTI measurements of addicted-like and non-addicted-like rats, several brain regions showed alteration in the DTI parameters in the GM, analyzed by the voxel-wise test. Clear interpretation and significance of each DTI values is yet to be clarified. Nevertheless, it has been proposed that FA is sensitive to microstructural changes, while other DTI parameters can measure membrane density and changes in axonal diameters. FA is the most common measurement used in DTI studies and, in our study, 0crit group showed higher FA in the zona incerta (ZI) in comparison to 3crit rats. The increase in FA indicates a diffusion profile along the fiber tracts in this brain region. ZI is found adjacent to subthalamic nucleus and it is suggested to regulate the locomotor activity (Mogenson et al., 1985; Supko et al., 1991) and the communications between posteromedial thalamus and superior colliculus. Superior colliculus affects interactions between dorsolateral striatum and posteromedial thalamus, halting ongoing behaviors in order to adapt motor actions to unexpected sensory events (Watson et al., 2015). The

FA in GM is expected to be lower as water diffusion is more isotropic due to diverse neuronal communications. Therefore, the higher FA values in ZI in the 0crit group indicate that water diffusivity is more in one orientation, which may suggest increased communication with other brain structure, such as the superior colliculus. Although speculative, this may partially explain the controlled drug use in 0crit group.

In conclusion, the present preliminary results in cocaine addicted-like and non-addicted-like rats indicate that GM volume in several brain regions increased significantly in the addicted-like rats over the course of drug use, while non-addicted-like rats showed normal development of GM volume through age. In addition, analysis of the white matter integrity revealed that non-addicted-like rats showed higher anisotropic movement of water in the ZI, a brain region that regulates brain structures modulating adaptive behavior. Increased communication between those regions may reflect efficient responses towards sensory stimuli, such as cocaine, resulting in more behavioral flexibility to adapt the cocaine-seeking responses. Further analyses are needed to confirm this hypothesis.

2.2 Positron Emission Tomography

Positron emission tomography [PET] is a highly sensitive imaging technique that enables *in vivo* assessment of biochemical and pharmacological changes in a subject (James and Gambhir, 2012). PET uses the radioactive isotopes properties, such as short-lived radionuclides ^{18}F ($t_{½}$ = 109.8 min) and ^{76}Br ($t_{1/2}$ = 16.2 h), or ultra-short-lived ^{11}C ($t_{1/2}$ = 20.3 min) and ^{15}O ($t_{1/2}$ = 2.04 min), to obtain and reconstruct the imaging. The nuclei of the radioactive isotopes have an excess of protons, which decays via positron emission. The newly formed positron is then ejected from the nucleus and collides with an electron in the surrounding tissue, losing rapidly the kinetic energy. This phenomenon is known as annihilation. The combined mass of the positron and electron produces emission of two photons, traveling at opposite directions (180°) from one another. PET detectors, which have a shape of a closed ring around the subject to be imaged, detect the annihilation events (gamma-rays). The electrical signals are then converted into sinograms that are used to reconstruct the tomographic images.

In our study, the radiotracer ^{18}F-fluorodeoxyglucose (^{18}F-FDG) was used to identify brain glucose metabolism associated with cocaine addiction. ^{18}F-FDG was first synthetized by Ido et al. (1978), and is an analog of the glucose molecule (Fig. 12), where the 2-carbon hydroxyl group of glucose was substituted for a fluorine atom (^{18}F). Similar to glucose, ^{18}F-FDG is uptake by high-glucose-using cells, including brain, cancer cells, kidney and adipocytes, and go through the first step of glycolysis metabolism. In this first metabolic step, it is phosphorylated by hexokinase II, forming ^{18}F-FDG-6-PO_4. However, due to the absence of the required 2-carbon hydroxyl to continue the metabolic steps as well as the presence of phosphate, it cannot be further metabolized or leave the cell, becoming metabolically trapped intracellularly. The accumulation of the ^{18}F-FDG over time leads to signal amplification and is thought to reflect more generally, the metabolic requirements of cells (James and Gambhir, 2012).

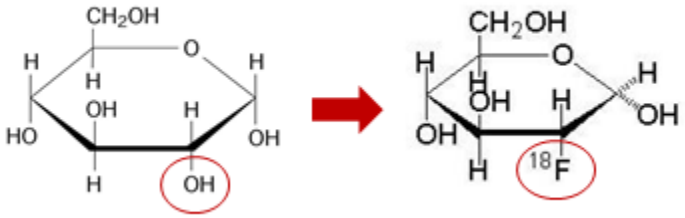


Figure 12 Analogy between glucose and fluorodeoxyglucose molecules
The hydroxyl of the 2-carbon is substituted by fluorine atom in the fluorodeoxyglucose molecule.

Previous studies using FDG-PET have demonstrated abnormal brain glucose metabolism in cocaine addiction and withdrawal. Specifically, acute withdrawal of cocaine-abusers was associated with higher glucose metabolic rate compared to drug-naïve controls or cocaine abusers in late withdrawal (Jentsch et al., 2002; Volkow et al., 1991). Conversely, cocaine abstinence was associated with decreased cortical glucose metabolic rate (Volkow et al., 1992), and the severity of cocaine abuse was negatively correlated with glucose metabolism (Moreno-Lopez et al., 2012b; Szumlinski et al.; Volkow et al., 1991). In other clinical studies evaluating relapse triggered by drug-related cues (Bonson et al., 2002; Grant et al., 1996; Volkow et al., 2011;

Volkow et al., 2010; Wang et al., 1999) or cocaine challenge (London et al., 1990) found reduction of glucose utilization. The divergent results reported in clinical studies may have been produced by the different stages of substance use disorder in which the cocaine abuser was found at the moment of the study.

Preclinical cross-sectional studies can help to overcome these limitations. Hence we performed a FDG-PET study to investigate differences in brain glucose metabolic rate of cocaine addicted-like (3crit), non-addicted-like (0crit) and drug-naïve controls. Each animal was scanned under baseline, cocaine-challenge and yohimbine-challenge conditions in different scanning days. Yohimbine is an antagonist of α2-adrenergic receptor and facilitates sympathetic activation by increasing plasma norepinephrine (Shibao et al., 2010). Because stress can reinstate drug-seeking behavior, we investigated changes on the brain metabolism produced by yohimbine.

Material and Methods

Animals and cocaine self-administration

Animals were trained in the 0/3crit model of cocaine addiction as described in the section 2.1.

Drugs

Cocaine hydrochloride (Sigma Aldrich, Taufkirchen, Germany) was dissolved in sterile saline.

PET Acquisition and Processing

Three PET acquisitions were performed for each animal using [^{18}F]-Fluorodeoxyglucose combined with saline, cocaine (0.8 mg/kg) or yohimbine (0.5 mg/kg). For data correction on intra-individual blood glucose levels variation, blood glucose levels were measured in duplicate using a blood glucose device (GlucoCheck XL; Aktivimed GmbH, Rheine, Germany; 0.5 μL from tail blood). FDG (mean 28.25 ± 2.95 MBq; range between ~23 and 33 MBq) was administered through the catheter implanted intravenously in the rat, flushing it with heparinized solution before and after FDG administration. Animals were injected during awake-state and following 30 min from the radiotracer injection, they were anesthetized with isoflurane (2–3.5% delivered at 2.5 L/min) and kept under anesthesia until the end of the procedure. PET scan was

acquired with tri-modal Bruker Albira small-animal PET/SPECT/CT (Bruker Biospin GmbH), starting at 40 min post-injection for 30 min, followed by CT image. Maximum likelihood expectation maximization algorithm was used for PET reconstruction with a matrix size of 20 × 20 and a pixel size of 0.5 mm (12 iterations) with data output in kBq/cc, using the Albira Suite Reconstructor software (Bruker Biospin MRI GmbH, Ettlingen, Germany). Data was corrected for scattering, attenuation, and dead-time. Images were normalized and transformed to a standard FDG-PET template, using PMOD v3.6 (PMOD Technologies, Zurich, Switzerland) for brain normalization.

Image analysis

Reconstructed PET images showing cerebral metabolic rate of glucose (CMRGlu) were spatially normalized to the FDG-PET template for rats by affine transformation. Standardized uptake values (SUV) were then calculated. To adjust inter-individual differences in global uptake due to biological and physical variabilities, SUV maps values were scaled by adjusting the mean based on the average of SUV within the entire brain mask that excludes regions outside the brain (SUVR). SUVR equals the globally normalized CMRGlu by canceling the injected radiotracer and the body weight factors in the calculation of SUV. Fifty-eight predefined regions of interest (ROIs) encompassing the entire brain were defined and the regional mean SUVR was calculated.

Cocaine-induced reinstatement

Following the end of PET scanning procedures, rats were reintroduced for a minimum of seven CSA sessions prior to reinstatement tests. Two reinstatement tests were performed, each lasting 210 min. In the first test, following 90-min extinction period, rats received four intravenous boluses (20, 40, 80, and 160 μL) of vehicle every 30 min. In the second test, the same schedule was applied for cocaine infusions, at the doses of 0.4, 0.8, 1.8, and 3.2 mg/kg, respectively. Number of nose-pokes were recorded, but had no scheduled consequences.

Yohimbine-Induced Reinstatement

After cocaine-induced reinstatement, another baseline CSA training was performed for a week by the animals. Similar to cocaine-induced reinstatement, it was a two-day protocol, lasting 180 min

each. However, a single dose of the drug was used. Following 90 min extinction, rats received an intravenous bolus of vehicle (80 µL), followed by another 90 min of reinstatement. In the second test, the same schedule applied for an injection of a bolus of yohimbine solution (0.5 mg/kg). Number of nose-pokes were recorded, but had no scheduled consequences.

Statistical Analysis

Performance of 0crit (non-addicted-like) and 3crit (addicted-like) rats in each of the addiction-like criterion (persistence of cocaine-seeking, motivation for cocaine-taking, and resistance to punishment) and total number of cocaine infusions were analyzed by Student's t-test. Reinstatement tests were analyzed with three-way ANOVA [factors: groups, number of nose-pokes (active/ inactive), and 30 min time bins], followed by Newman–Keuls post hoc analysis when appropriate. Regional brain glucose uptake was analyzed with a two-way mixed ANOVA. Statistical significance was set at $P < 0.05$, followed by post hoc pairwise comparison analysis.

Experimental Timeline

After characterization of addiction-like behaviors, 0crit, 3crit and AC rats were selected for PET acquisitions (n= 8/treatment group). Baseline training was maintained until the PET acquisitions and the scans were counter-balanced for saline, cocaine and yohimbine administration. Following completion of PET scanning, rats were reintroduced for CSA training prior to cocaine- and yohimbine-induced reinstatement tests. Measurements were always held in the morning, during their dark phase. Fasting period of 12 h were ensured to avoid pre-scan glucose level corrections. The inter-scan period was set for at least 48h to diminish influences of previously FDG variations in brain uptake values caused by changes in blood glucose level.

Results

Dr. Alejandro Cosa-Linan analyzed the entire neuroimaging data.

Characterization of Addiction-Like Behavior

Out of 48 rats that initiated in the CSA training, 5 rats were excluded for catheter problems. Within the 43 remaining animals, 15 rats were 0crit, 11 rats were 1crit, 9 rats were 2crit, and 8 rats were 3crit. The 0crit were those rats scoring below the 60th percentile in all addiction criteria and categorized as non-addict-like, whereas 3crit scored above the 60th percentile in all criterion and categorized as addict-like. The 3crit group was significantly different from the 0crit group in all criteria: persistence of drug-seeking [$t(14) = -5.04$, $P = 0.00018$; Fig. 13A], motivation for cocaine [$t(14) = -7.11$, $P = 0.000005$; Fig. 13B] and resistance to punishment [$t(14) = -5.07$, $P < 0.00017$; Fig. 13C]. However, 0crit and 3crit did not differ in total number of cocaine infusions [$t(14) = -0.2$, $P = 0.84$; Fig. 13D].

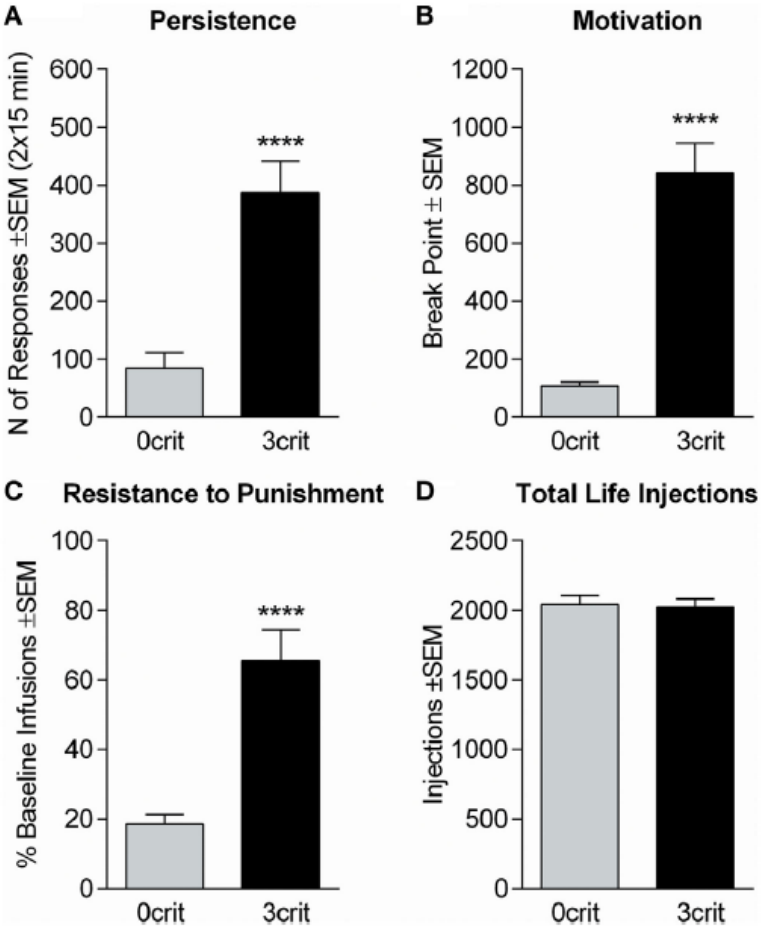


Figure 13. Characterization of addiction-like behaviors.
Behavioral characterization of 0crit and 3crit rats selected for PET acquisitions. (A) Persistence of cocaine-seeking, (B) motivation for cocaine intake, (C) resistance to punishment, (D) total

number of cocaine infusions (n=8/ group). Data is shown as mean ± SEM; ****P < 0.0001.

Group and Drug-Induced Differences in SUV Reveal Potential Biomarkers

One 3crit rat died during anesthesia; therefore seven 3crit rats underwent the PET scanning. There was no group (F = 0.513, P = 0.607), drug (F = 0.514, P = 0.482) or drug interactions (F = 1.2, P = 0.324) (Fig. 14 A, B) in the averaged whole brain uptake under baseline and cocaine conditions. The comparison of the average whole brain uptake under baseline and yohimbine conditions, only the drug effect was significant (F = 17.1, P = 0.001), suggesting that the effect of yohimbine was group independent (Fig. 14B).

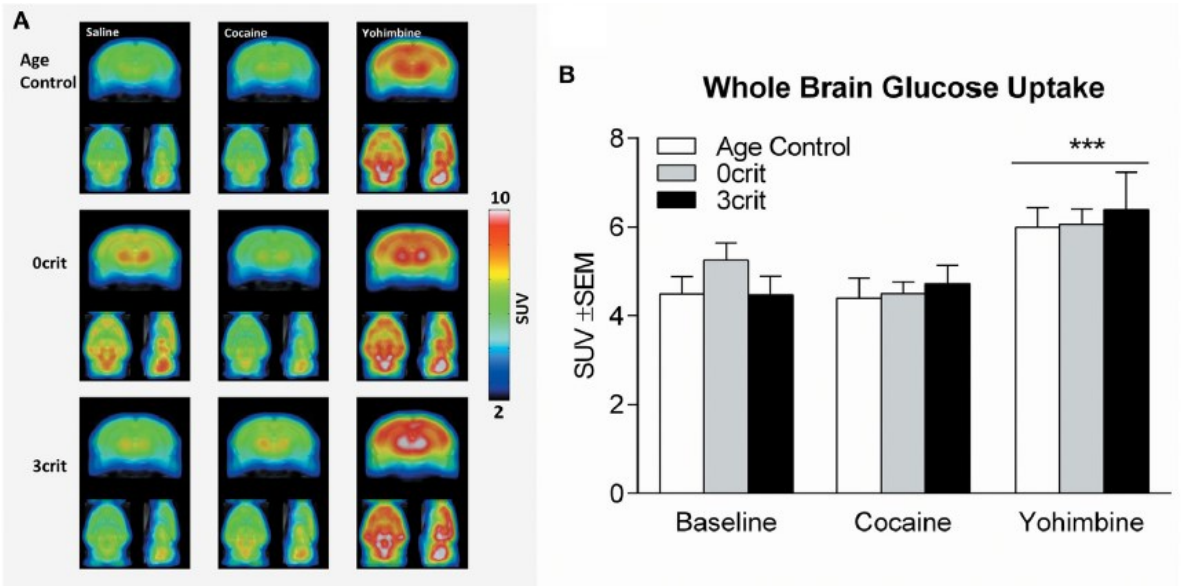


Figure 14. Whole brain glucose uptake in Age Control, 0crit and 3crit rats.
Average reconstructions of FDG uptake in Age Control, 0crit, and 3crit rats under baseline, cocaine or yohimbine conditions (A, left panel, upper, middle and lower rows, respectively). Standardized uptake value (SUV) of the whole brain after FDG injection in combination with saline, cocaine or yohimbine in Age Control, 0crit, and 3crit rats (B, right panel). ***P < 0.001 vs baseline scan

SUV differences in Baseline scanning

In both left and right mPFC and in the right CPu a higher SUV was found in the 0crit compared to AC rats. In 3crit rats, higher metabolic activity in these regions was not found. In the left

somatosensory cortex, left visual cortex, left motor cortex, and left frontal association cortex, lower baseline SUV was found in 3crit compared to AC and 0crit rats (Fig. 15).

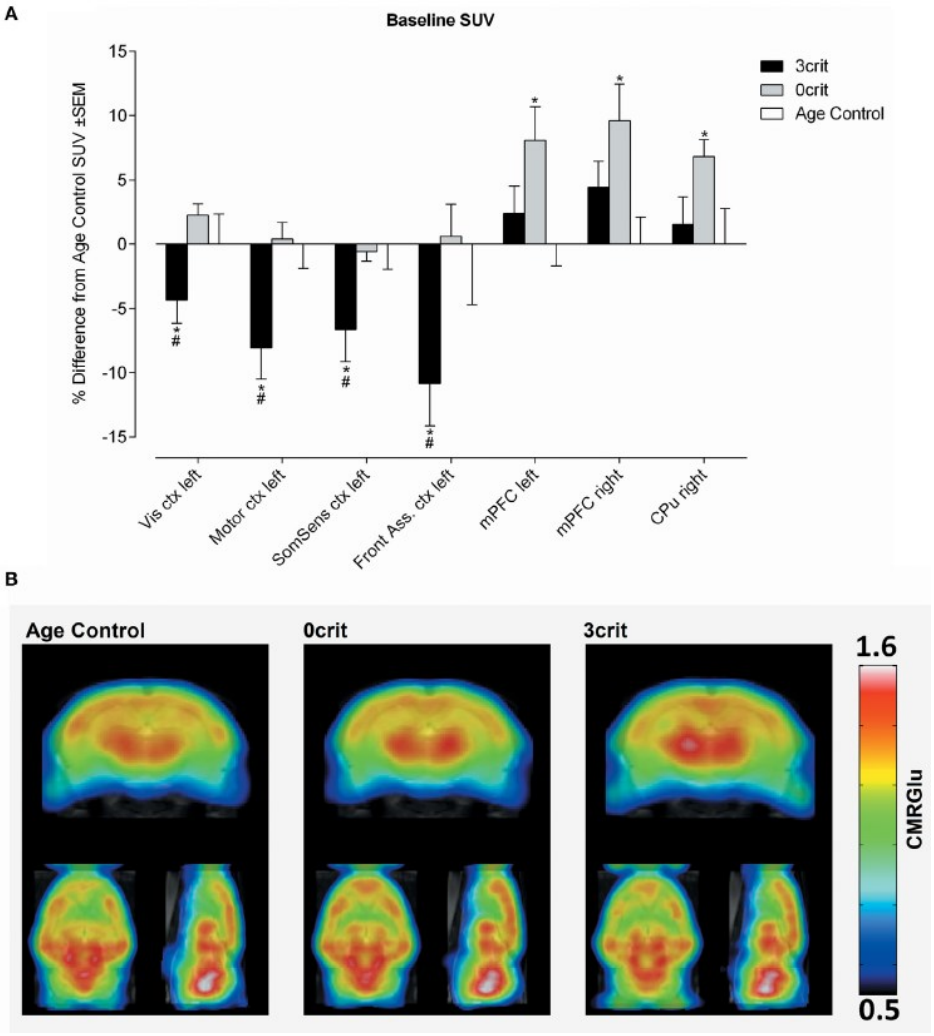


Figure 15 Baseline SUV in AC, 0crit and 3crit rats.
Local quantification of metabolic activity in baseline condition. (A) Baseline SUV expressed as relative difference after saline bolus injection from Age Control. 3crit rats showed lower SUV in several cortices; 0crit showed higher SUV in mPFM and CPu compared to Age Control rats. (B) Metabolic maps representing baseline cerebral metabolic rate of glucose (CMRGlu) in Age Control, 0crit, and 3crit rats. *P < 0.05 vs Age Control, #P < 0.05 vs 0crit. CPu, caudate putamen; mPFC, medial prefrontal cortex; Front Ass ctx, frontal association cortex; SomSens ctx, somatosensory cortex; Motor ctx, motor cortex; Vis ctx, visual cortex.

Cocaine-Induced Changes in SUV

As shown in Figure 16, injection of a cocaine bolus produced bilaterally increase in glucose uptake in the mPFC, and a decrease of glucose uptake in the hypothalamus in Age control rats, which significantly differed from 3crit animals. No changes in glucose uptake were found in both 0crit and 3crit animals.

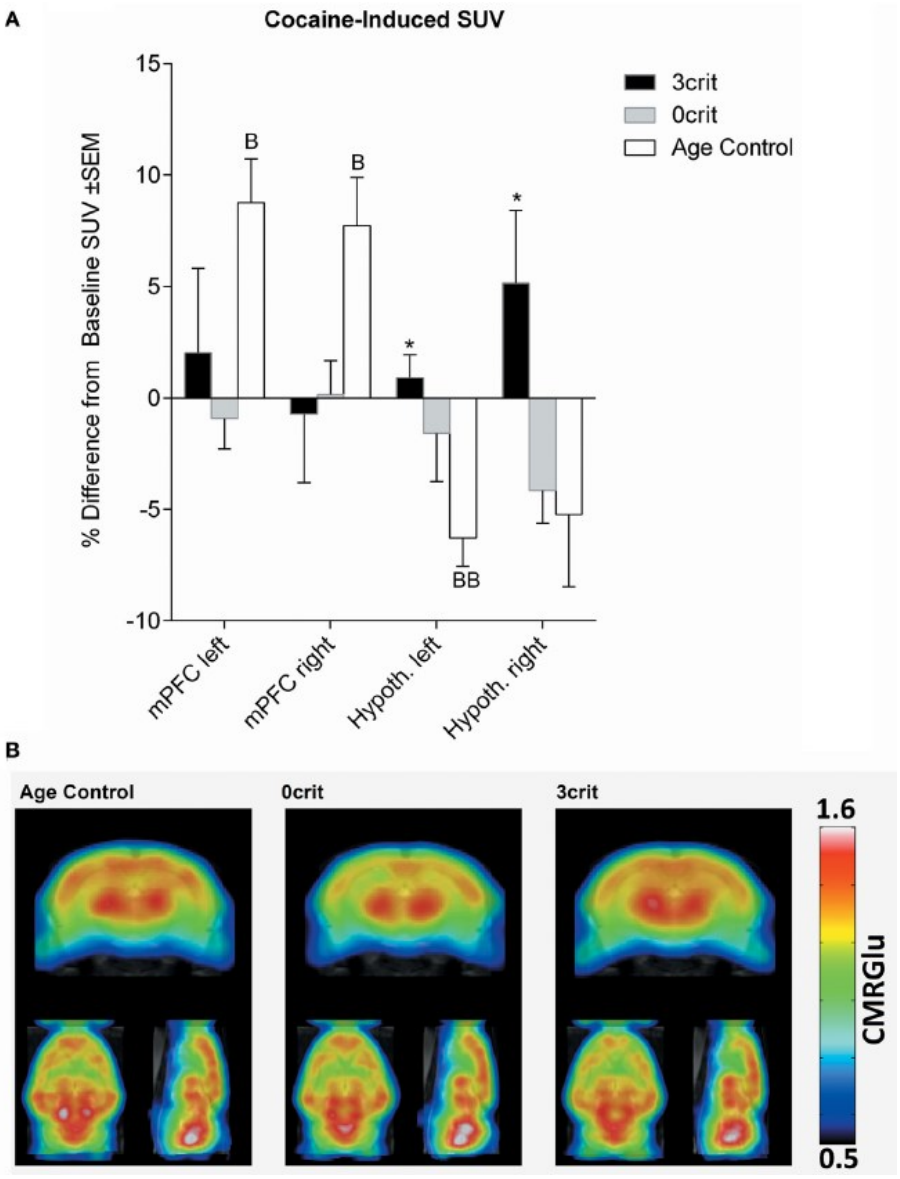


Figure 16 Local quantification of metabolic activity after cocaine bolus injection.
(A) Cocaine-induced SUV expressed as relative difference from baseline. Increased glucose uptake in mPFC and decreased in the hypothalamus of Age Control rats. (B) Metabolic maps representing cocaine-induced cerebral metabolic rate of glucose (CMRGlu) in Age Control, 0crit,

and 3crit rats. *P < 0.05 vs Age Control, B P < 0.05 and BB p < 0.01 vs Baseline SUV. mPFC, medial prefrontal cortex; Hypoth, hypothalamus.

Yohimbine-Induced Changes in SUV

As shown in Fig. 17, injection of a yohimbine bolus induced a significant SUV increase in the cerebellum, medulla, left retrosplenial cortex, and left ventral tegmental area (VTA), and a decrease in the septum in AC rats. This effect differed significantly from 3crit animals in the septum, medulla and cerebellum. Yohimbine did not produce changes in SUV in both 0crit and 3crit animals.

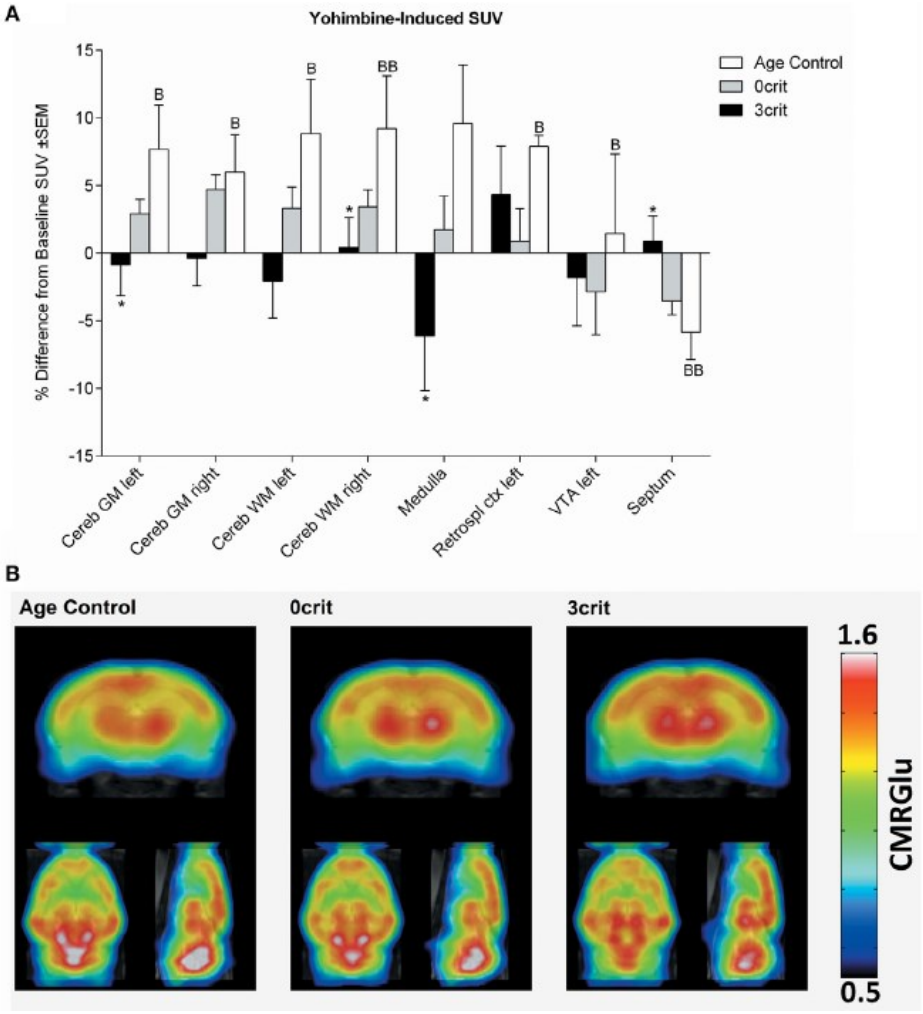


Figure 17 Local quantification of metabolic activity after yohimbine bolus injection.
(A) Yohimbine-induced SUV expressed as relative difference from baseline. Yohimbine increased SUV in several brain areas and decreased in the septum of Age Control rats. (B)

Metabolic maps representing yohimbine-induced cerebral metabolic rate of glucose (CMRGlu) in Age Control, 0crit, and 3crit rats. *P < 0.05 vs Age Control, B P < 0.05, and BB P < 0.01 vs Baseline SUV. VTA, ventral tegmental area; Retrospl ctx, retrosplenial cortex; Cereb WM, white matter of cerebellum; Cereb GM, Gray matter of cerebellum.

Correlation between Behavioral Addiction Sub-Dimensions and Regional Glucose Uptake

Correlations surviving Bonferroni analysis were almost exclusively associated with the metabolic activity of 0crit group. Specifically, under baseline conditions the BP positively correlated with SUV in the VTA (left r2 = 0.7379, P = 0.006; right r2 = 0.7885, P = 0.003). Cocaine-induced SUV in the right frontal association (r2 = 0.8154, P = 0.002), right medial prefrontal (r2 = 0.8118, P = 0.002), and right orbitofrontal (r2 = 0.7534, P = 0.005) cortices correlated positively with persistence of cocaine-seeking. Yohimbine-induced SUV within the cingulate cortex (left r2 = 0.7569, P = 0.005; right r2 = 0.7413, P = 0.006) and anterodorsal hippocampus (r2 = 0.8686, P = 0.001) correlated negatively with resistance to punishment. In the 3crit condition, only cocaine-induced SUV in the right VTA correlated negatively with resistance to punishment (r2 = 0.8968, P = 0.001) and survived Bonferroni correction.

Yohimbine-Induced Reinstatement

Yohimbine-induced reinstatement (Fig. 18 C) revealed no significant interactions between factors: nose-pokes vs time bins vs group interaction [$F_{(3,33)}$ = 1.18; P > 0.05]. However, there was an effect of the time bins [$F_{(3,33)}$ = 3.11; P < 0.05]. Therefore, we run anyway a post hoc analysis that indicated a significant increase in active nose-poke compared to extinction (P < 0.01) during the third reinstatement time bin by 3crit rats. In addition, in contrast to 0crit rats, during the third reinstatement time bin, active pokes were significantly higher than inactive pokes in 3crit rats. Thus, data suggested a tendency of 3-crit animals to exhibit enhanced yohimbine-induced reinstatement responding. However, this result should be taken cautiously as only five 3crit rats were tested and yohimbine usually produces great variability, as was the case here.

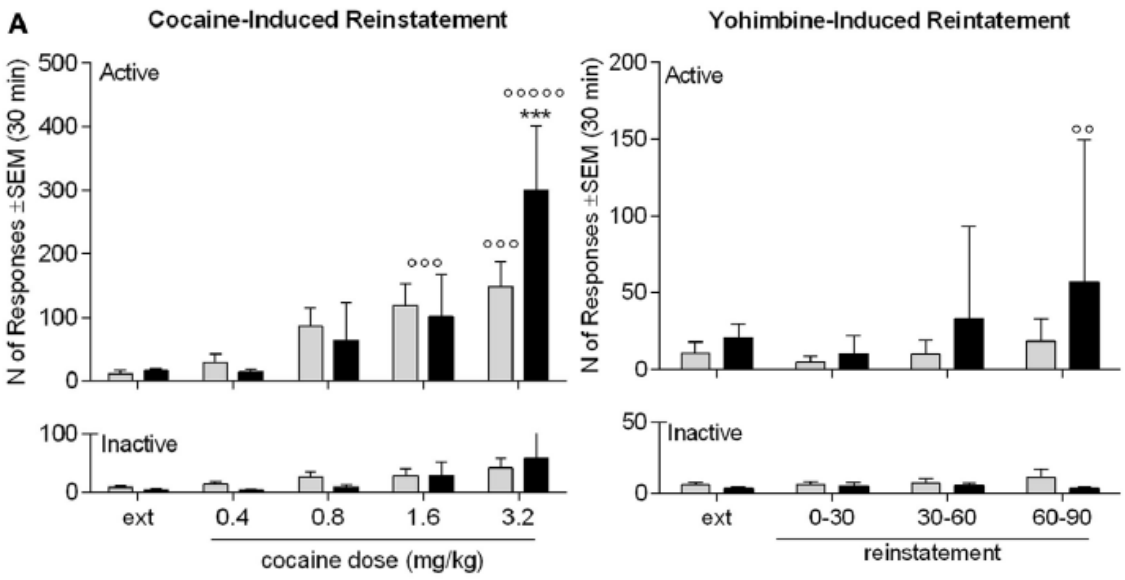


Figure 18. Cocaine (A) and yohimbine (B) induced reinstatement.
Cocaine (A) and yohimbine-induced (B) reinstatement in 0crit (n = 8) and 3crit (n = 5) rats. (A) Groups did not differ in number of active nose-pokes during the last 30 min of extinction (ext). The following 120 min of reinstatement is shown as 30 min time bins, each followed by infusion of cocaine doses (0.4, 0.8, 1.6, 3.2 mg/kg). The dose of 1.6 mg/kg reinstated both 0crit and 3crit rats, whereas the dose of 3.2 mg/kg selectively increased in 3crit rats. (B) Groups neither differed in number of active nose-pokes during the last 30 min of extinction (ext) nor following injection of yohimbine (0.5 mg/kg). Data are shown as mean ± SEM; ***P < 0.001 vs 1.6 mg/kg dose; °°P < 0.01 and °°°P < 0.001 vs extinction.

Discussion

The present FDG-PET study shows reduced glucose uptake in cortical areas, including motor, visual, somatosensory and parietal association cortices in brains of cocaine addicted-like (3crit) rats compared to control rats, whereas higher glucose uptake in mPFC and CPu in non-addicted like (0crit) rats compared to control rats during baseline acquisition. Cocaine and yohimbine challenge increased glucose uptake in the control rats in several brain regions, however this effect was not found in 0crit and 3crit.

Identification of biomarkers in disease states can be very helpful to prevent progression of the pathology as well as adequate treatment for the stage in which the disease is found. In drug addiction, there is considerable interest in prolonging periods of abstinence and prevent relapse.

However, little evidence has been found that indicate biomarkers for addiction pathology or resilience. In our translational approach with cocaine addiction model we have the advantage to control environmental conditions, age, and genetic background in order to investigate biological features associated with each stage of the disease in animals that show similar levels of drug intake. In our cocaine non-addicted-like rats (0crit) we found higher cerebral metabolic rate of glucose (CMRGlu) in the mPFC and CPu compared to the control group during the baseline acquisition. mPFC plays an important role in higher cognitive tasks, including inhibitory control and action-outcome evaluation. Therefore, the baseline increased CMRGlu in this region in 0crit rats could indicate compensatory effects to counteract the impairments caused by chronic drug use, enhancing the behavioral control. Coherent with this hypothesis, activation of prelimbic cortex with optogenetic reversed the compulsive cocaine self-administration in addicted-like rats (Chen et al., 2013). Clinical studies have also shown that drug addicted subjects display hypoactivity in the frontal cortex of the brain, which was correlated with impairments in inhibitory tasks (Dalley et al., 2004; Goldstein and Volkow, 2011). Therefore, it appears that the enhanced PFC activity in non-addicted rats could indicate a response to produce controlled drug use, while the loss of metabolic activity in this area could be associated with addiction.

Cocaine challenge increased CMRGlu in the mPFC in the control group, but had no such effects in both non-addicted-like and addicted-like rats. This might be explained by the development of tolerance to cocaine effects (Hammer and Cooke, 1994), a common characteristic displayed by drug users (Emmett-Oglesby et al., 1993). Interestingly, in the control group cocaine challenge significantly increased the metabolic glucose activity in the distal end of the mesocorticolimbic dopaminergic neurons, the mPFC. Furthermore, no difference in the activation of NAc, the central region for drug rewarding effects, was found in comparison with cocaine experience rats. This result suggests that chronic cocaine use produce primarily changes in the mPFC and other brain areas, including the NAc. Similar to cocaine challenge, yohimbine administration, a pharmacological stressor, did not produce significant changes in CMRGlu in both non-addicted-like and addicted-like rats. However, in the control group yohimbine increased CMRGlu in several brain areas, except in septum. This is an intriguing result because it suggests that chronic cocaine use also affects the norepinephrine system. Nevertheless, despite the lack of differences

in the glucose uptake between 0crit and 3crit following cocaine or yohimbine challenge, 3crit rats showed higher number of active nose-pokes during cocaine-priming reinstatement (at the dose 3.2 mg/kg) compared to 0crit. Conversely, yohimbine-induced reinstatement was similar between the groups.

In conclusion, the higher baseline glucose utilization by mPFC and CPu in 0crit rats may suggest the maintenance of behavioral control over the drug taking and seeking. This brain adaptation was not present in 3crit rats, suggesting potential biomarkers for resilience to addiction. Further studies are still warranted to investigate the molecular mechanisms that provide increased mPFC and CPu in modulating the behavioral control.

Chapter 3: Neuronal changes in the cocaine addiction

The capacity of the brain to adapt to different situations and environments depends on plasticity of synaptic connections. Plasticity requires an orchestrated synthesis of specific mRNAs and proteins that facilitate molecular and structural changes at the synapse (Bramham et al., 2010; Havik et al., 2007; Lee et al., 2005; Liao et al., 2007; Park et al., 2006; Wibrand et al., 2006; Wibrand et al., 2010). As a result, a neuron has the ability to modulate synaptic connections in response to input it receives, shaping emotional responses, memory formation and cognitive flexibility. Several mental disorders, such as autism, Alzheimer's disease and drug addiction, have been associated to aberrant synaptic plasticity (Minatohara et al., 2015). The biological mechanisms of synaptic plasticity have been extensively studied in long-term depression (LTD) and long-term potentiation (LTP) mediated through gene expression and protein synthesis (Alberini, 2009; Alberini and Kandel, 2014; Alberini et al., 2006; Minatohara et al., 2015; Miyashita et al., 2008; Okuno, 2011; Wang and Morris, 2010).

Recent studies have conveyed to the role of immediate early genes (IEGs) in mediating stimulus-induced neuronal plasticity (Fosnaugh et al., 1995; Goelet et al., 1986; Morgan and Curran, 1989; Sheng and Greenberg, 1990; Worley et al., 1990). IEGs are a subset of genes that is induced by neural activity (e.g., via NMDA receptor activation and neuronal calcium influx) during behavioral and sensory experience (Heroux et al., 2018; Minatohara et al., 2015). Its transcription is transient, and rapidly increases following neuronal activity associated with induction of synaptic plasticity (Cole et al., 1989) and with attentive brain states (Guzowski et al., 2000; Guzowski et al., 2001; Tzingounis and Nicoll, 2006; Wallace et al., 1998). IEGs expressions are extremely low (generally below detection thresholds) in resting animals (Guzowski et al., 2005) and therefore IEG expression can be ideally used to map behaviorally relevant circuits in experimental animals (Guzowski et al., 2005). Among several IEGs that is expressed in several brain regions, such as Arc, c-fos, egr-1, and homer1a, Arc particularly has a unique characteristic that both of its mRNA and protein can be found in the dendritic compartments of neurons (Guzowski et al., 2005; Minatohara et al., 2015; Vazdarjanova et al., 2002; Wilkerson et al., 2018).

Arc expression occurs minutes after neuronal activation triggered by behavioral events. Its mRNA is expressed in the nucleus and trafficked to the cytoplasm and dendrites. The mRNA accumulates at sites of synaptic activity and undergoes local translation (Link et al., 1995; Lyford et al., 1995; Moga et al., 2004; Rodriguez et al., 2005; Steward et al., 1998; Yin et al., 2002). Because Arc is primarily expressed in neurons (Rao et al., 2006; Vazdarjanova et al., 2006, but see Rodriguez et al., 2005) and is localized in dendrites that receive active synaptic stimulation (Dynes and Steward, 2007; Lyford et al., 1995; Moga et al., 2004; Steward et al., 1998; Steward and Worley, 2001), it has been proposed that Arc may mediate cytoskeleton changes underlying stimulus-induced neuronal plasticity.

Actin cytoskeleton plays an important role in regulating neuronal responsiveness (Fosnaugh et al., 1995; Johnson and Byerly, 1993; Rosenmund and Westbrook, 1993), and drugs of abuse, such as cocaine, appear to elicit changes in the cellular cytoskeleton (Nestler, 1993). More specifically, drugs of abuse affect the pattern of neurofilament expression and the rate of axonal transport (Beitner-Johnson et al., 1992; Nestler, 1993). Furthermore, it has been suggested that Arc may be involved in activity-dependent depression of excitatory synaptic transmission observed in mGluR-dependent LTD as well as in homeostatic plasticity (Bramham et al., 2010). The interaction of Arc with components of the endocytic machinery (dynamin and endophilin 2/3) could be leading to internalization of surface AMPAR-type glutamate receptors (Chowdhury et al., 2006; Park et al., 2008; Rial Verde et al., 2006; Shepherd et al., 2006; Waung et al., 2008). Dysfunction of LTD has been previously reported in addicted-like rats. Impairment of LTD was found in the mPFC as well as in NAc core of addicted-like rats compared to non-addicted and controls rats (Kasanetz et al., 2010; Kasanetz et al., 2013). Other studies have also demonstrated that during the course of cocaine use, the escalating behavioral effects in cocaine self-administration correlated with dendritic changes in PFC and NAc core (Kolb and Gibb, 2015; Robinson and Kolb, 1999, 2004). These changes in PFC neurons were rapidly detectable, in terms of few hours following cocaine injection (Munoz-Cuevas et al., 2013). Chronic cocaine or amphetamine exposure also appear to increase dendritic length and spine density in those brain regions, and to decrease in OFC (Crombag et al., 2005). Taken these studies together, it appears

that Arc not only is an indicative of neuronal activity mediated by stimulus, but also chronic drug use could alter its function in coordinating the downstream molecular signaling, changing the number of dendritic spines and consequently affecting the brain function.

Based on the facts mentioned above, our aim was to assess whether Arc would differently express in several brain regions linked to addiction, such as the compartments of PFC, ventral and dorsal striatum, and AMY during the cue-induced reinstatement of cocaine addicted-like and non-addicted-like rats. To analyze the dendritic spines, a synthetic activity-dependent E-SARE virus was used (Kawashima et al., 2013) to label only the activated neurons during the cue-induced reinstatement. The synaptic activity-responsive element (Bassareo and Di Chiara) is located at ~7 kb upstream of the transcription initiation site of Arc (Inoue et al., 2010). It possesses a strong enhancer activity that is sensitive to synaptic stimulus, and the enhanced-SARE (E-SARE) is an enhanced version of the SARE promoter, which drives downstream expression at higher levels than the IEG promoter (Kawashima et al., 2013).

Material and methods

Animals and cocaine self-administration

Animals were trained in the 0/3crit model of cocaine addiction. In the present study, addicted-like (3crit) and non-addicted-like (0crit) rats were used for assessment of Arc expression (n=6-7/ group). To spine density analysis with E-SARE, two 1crit and two 2crit rats were used for a preliminary analysis.

Extinction

Extinction training started on the next day after the last CSA training session (~65 CSA). During extinction sessions, blue cue-light and contingent white cue-light were withdrawn. Nose-pokes were recorded throughout the 2h extinction session; however it did not result in cocaine infusion. Extinction training continued until responses reduced to about 20% of the baseline conditioning CSA sessions.

Cue-induced reinstatement

Reinstatement test last for 2h and rats were exposed to the same conditions as during the conditioning phase, except that the cocaine was not available. Operant chamber was constantly illuminated by the blue cue light as a contextual stimulus for cocaine availability and the contingent white cue-light was delivered at the completion of FR5. The number of nose-poking in both active and inactive holes was recorded throughout the test.

For Arc assessment, reinstatement was performed on the second day after the final extinction training.

For E-SARE study, animals were retrained for a week in the CSA following 3-4 weeks from stereotaxic surgery. On the next day after the last CSA session, animals underwent extinction sessions. Reinstatement test was performed on the third day after the final extinction training due to the long-lasting GFP expression.

Immunohistochemistry

Arc expression was analyzed in several brain regions of cocaine addict-like and non-addict-like rats. Immediately after the end of reinstatement test, rats were deeply anesthetized with isoflurane and intracardially perfused with 150-200 ml of 1× PBS pH 7.4 followed by 100 ml of 4% paraformaldehyde (PFA). Brains were then post-fixed overnight for 24 h at 4°C in 4% PFA. Coronal sections of 55 μm thickness were made with vibratome and post-processed for free-floating immunohistochemistry (IHC).

Slices were washed three times in 1× TBS and thereafter incubated for 1h in blocking solution, containing 7.5% donkey serum, 2.5% BSA and 0.2% Triton X-100 in 1× TBS. Anti-Arc antibody (rabbit, 1:1000; Synaptic System, Goettingen, Germany) was incubated overnight in blocking solution at 4°C. Sections were then washed three times in 1× TBS and incubated with the secondary antibody Alexa-Fluor 488-labeled donkey anti-rabbit (1:200) in TBS-Triton for 1h. Slices were washed two times in 1x TBS and incubated with TOTO-3 (1:2000) in TBS-Tx for 5 min followed by another washing with 1x TBS. TOTO-3 was used to stain the nucleus of all cells for quantification purposes. TOTO-3 shows far-red fluorescence with excitation and emission around 640 nm and 660 nm, respectively. Slices were mounted in glass slides with Immumount (Fischer-Scientific, Hampshire, United States) and images acquired with in confocal microscope.

Confocal microscope Leica TCS SP2 (Leica Microsystems) was used. Images were obtained using a 63× oil-plan achromatic lens with an NA 1.3. Argon ion laser (458–514 nm), and HeNe lasers (633 nm) were used to acquire the images. Z-stacks were made every 0.99 μm for Arc quantification. Three to five images per hemisphere per region were acquired for each animal. Images were saved as Tagged Image File Format (TIFF) files.

Stereotaxic injections

E-SARE virus was kindly provided by Dr. Ana Oliveira, Heidelberg University, Germany.

Animals were deeply anesthetized with isoflurane (400 ml/L, induction: 5%, maintenance: 2-2.5%) and placed in a Kopf stereotaxic head-holder (David Kopf Instruments, Tujanga, CA, USA). Craniotomies were made directly above the target region of the brain. About 300 nl per hemisphere of the E-SARE virus (ITR--E-SARE--HA-GFP--Syn-Td--ITR) was injected in the Infralimbic Cortex (AP: +3.2; ML: ±0.5; DV: -5.8).

Dendritic spines density

Dendritic spines density was assessed in the brains of animals injected with E-SARE virus. Following cue-induced reinstatement test, animals were immediately perfused with 4% PFA and brains were post-fixed with 4% PFA overnight at 4°C. Coronal sections of 70 μm thicknesses were made with vibratome and brain slices were mounted with Immumount (Fischer-Scientific, Hampshire, United States). Images were acquired with confocal microscope, as above mentioned above. Argon ion laser (458–514 nm), and diode-pumped solid-state laser (561 nm) were used to acquire the images. Z-stacks were made every 0.15 μm for spines visualization and about 10 – 11 images were acquired per animal in the mPFC cortex.

Dendritic spines were counted only in the secondary dendrites with minimum of 20 μm length. Spine density was calculated as the number of spines/ μm.

Data analysis

Reinstatement data was analyzed by a three-way analysis of variance (ANOVA). The factors were groups vs. nose-pokes (active/inactive) vs. sessions (extinction vs. reinstatement). Arc-positive cells and TOTO-3 staining were counted manually with ImageJ software and the relative expression was calculated using the following equation: (Arc-positive cells/ total number of cells

counted with TOTO) *100. One-way ANOVA was used to analyze the Arc expression per region of interest. Dendritic spine density was counted manually with ImageJ and the Student t-test was used for analysis between groups. Whenever significant differences were found, a Bonferroni post-hoc test was used. The chosen level of significance was $P < 0.05$.

Results

Cue-induced reinstatement test of cocaine non-addicted-like and addicted-like rats

Following ~65 CSA training sessions, 0crit animals showed 293 ± 98 active nose-pokes and 3crit rats showed 712 ± 185 active nose pokes. Inactive nose-pokes were about 76 ± 19 and 121 ± 102 for 0crit and 3crit, respectively. Extinction (Ext) training started on the day after the last CSA session. Extinction was performed for 2h daily until animals reached the extinction criteria (<20% AP from baseline conditioning). Cue-induced reinstatement test (Reinst) was performed 48h after the last extinction sessions, and the reinstatement test lasted for 2h. ANOVA followed by post-hoc test revealed significance difference between Ext vs. Reinst [$F_{(3, 33)} = 24.08$, $P = 0.01$], indicating that both groups showed drug-seeking behavior. Immediately after the end of reinstatement test, animals were perfused and their brains collected for immunohistochemistry analysis.

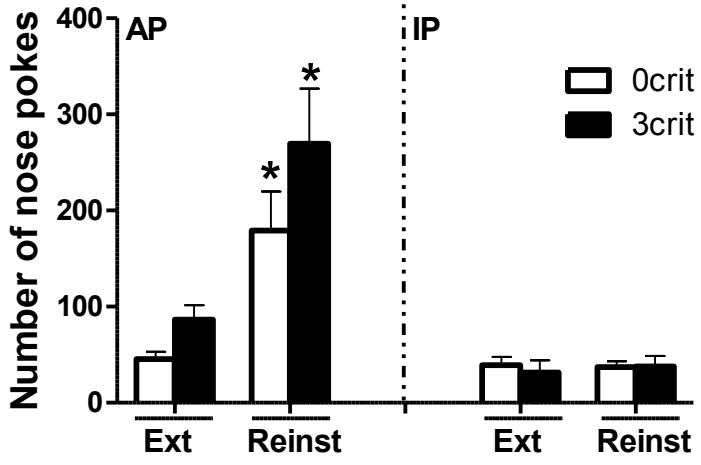


Figure 19. Cue-induced reinstatement test in addicted-like and non-addicted-like rats.
Bars represent number of active and inactive nose-pokes ± S.E.M. during the 2h reinstatement test elicited by cue. *difference vs. Ext ($P<0.01$)

Immunohistochemistry for analysis of Arc expression

Arc expression was analyzed in several brains regions related to cocaine addiction to identify differences in the neuronal activity between cocaine addicted-like and non-addicted-like animals. The chose regions were cingulate cortex (Cg), prelimbic (Prl), infralimbic (IFL), OFC, nucleus accumbens core (NaC) and shell (NaS), dorsal striatum (DS), and amygdala (AMY). Images of Arc and TOTO-3 IHC were obtained with confocal microscope, x63 magnification (Fig. 20).

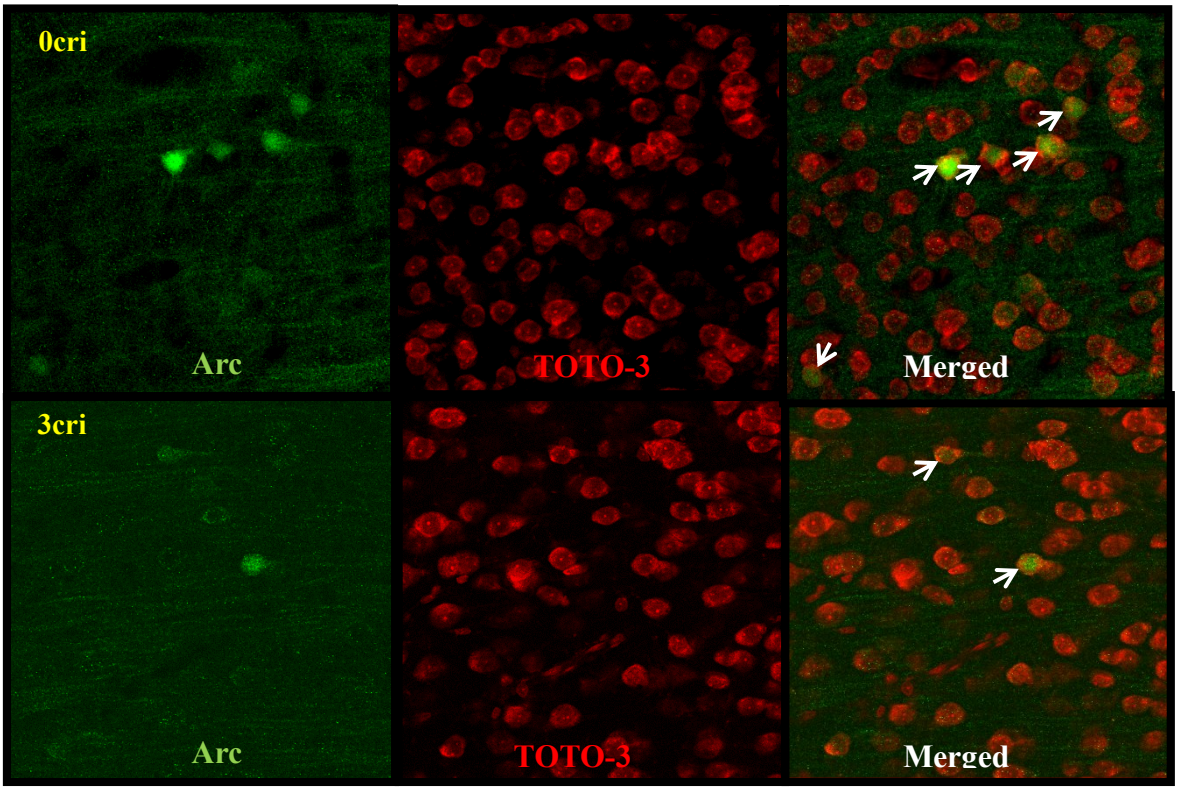


Figure 20 Arc and TOTO-3 staining in cocaine addicted-like and non-addicted-like rats following cocaine cue-induced reinstatement test
Pictures were collected in confocal microscope (x 63) for quantification.

Arc expression in the Infralimbic Cortex is reduced in addicted-like rats

Several brains regions that play a role in addiction behaviors were evaluated for Arc expression following cue-induced reinstatement test. ANOVA revealed significant difference in the relative

Arc expression [$F_{(7, 88)}$ = 12.72; P < 0.0001]. Post-hoc test showed Arc expression in the infralimbic cortex between 0crit and 3crit rats (Fig. 21).

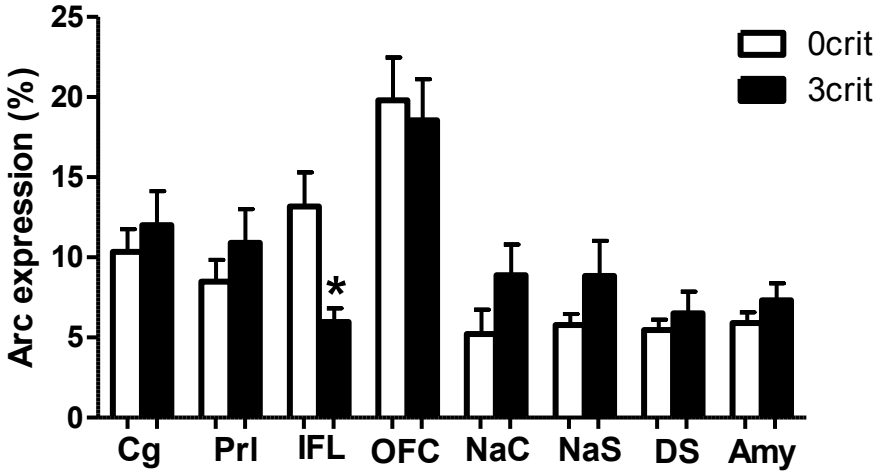


Figure 21. Relative Arc expression in different brain regions in addicted-like and non-addicted-like rats.
Arc expression was calculated in relation to number of cells in the acquired image (n = 6 – 7/ treatment group). Bars represent mean ± SEM. * different from 0crit within the region (P<0.05).

Correlation analysis between Arc expression and addiction-like behaviors

Considering that 3crit rats show higher cocaine-seeking behavior compared to 0crit, Pearson correlation test was used to investigate whether behavioral performance in the cue-induced reinstatement correlates with relative Arc expression. There was a significant correlation between Arc expression in NAc shell and performance in both persistence to seek cocaine (r = 0.74, P = 0.004) and resistance to punishment (r = 0.77, P = 0.012) tests. Resistance to punishment also correlated with Arc expression in amygdala (r = 0.56, P = 0.046). There was also correlation between brain regions. Arc expression in ACC correlated with expression in both OFC (r = 0.58, P = 0.039) and NAc shell (r = 0.57, P = 0.043). Although not significant, there was also a tendency for correlation between ACC and Prl cortex (r = 0.54, P = 0.056). The core compartment of NAc correlated with amygdala (r = 0.67, P = 0.013) and dorsal striatum (r = 0.52, P = 0.038), whereas the shell compartment correlated with dorsal striatum (r = 0.8, P = 0.001).

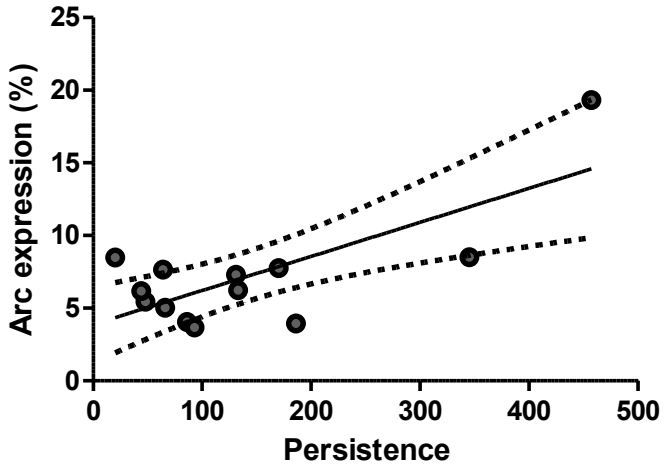


Figure 22. Pearson correlation between persistence and Arc expression in the Nac shell.
There was a positive correlation between performance in the persistence to seek cocaine and relative expression of Arc in the NAc shell (r = 0.74, P = 0.0036, n = 13).

Dendritic spine density

Following cue-induced reinstatement test, animals were immediately perfused with 4% PFA and brains sliced for further visualization of GFP expression from E-SARE virus. GFP expression was visible in the nucleus, dendrite and spines of activated neurons (Fig. 23). Spines were counted only in the secondary dendrites with minimum 20 μm length. Spine density was calculated as the number of spines/ μm of dendrites.

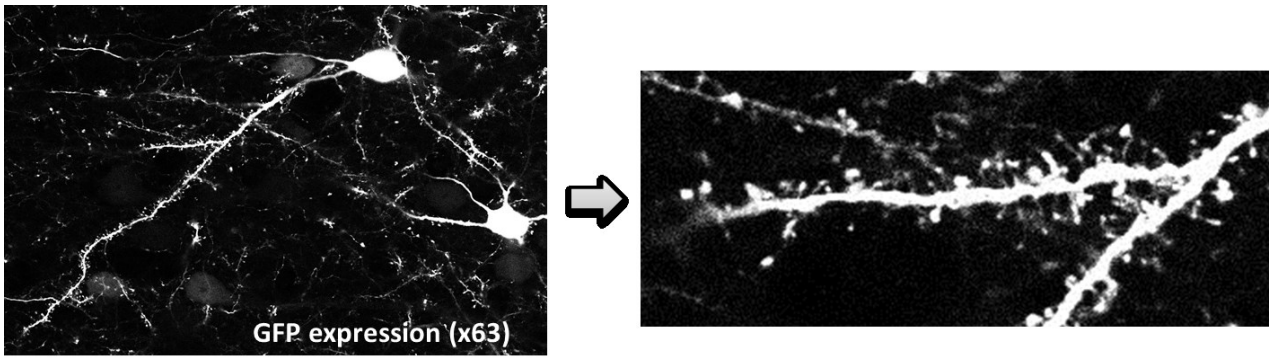


Figure 23. GFP expression from E-SARE virus in the activated neuron in the mPFC
GFP expression was visible in the nucleus, dendrites and spines. Images were acquired with confocal microscope (x 63) and analyzed with ImageJ.

Student t-test analysis showed no significant difference between 0crit and 3crit, likely caused by the small group size (n= 2/ group; P=0.11; Fig. 24). Spines were counted by a master student Nadine Stowasser.

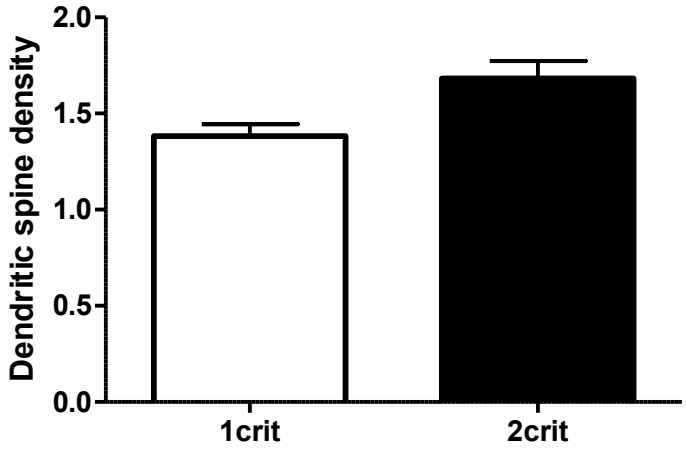


Figure 24 Dendritic spine density in 1crit and 2crit
Number of spines/ µm in the mPFC (prelimbic and infralimbic cortex) was analyzed in 1crit and 2crit rats (n=2/ group). Student t-test showed no significant difference (P=0.11). Data is shown as mean ± S.E.M. of dendritic spine density

Discussion

The present study shows that relative Arc expression following cue-elicited reinstatement was significantly reduced in the infralimbic cortex in the 3crit group compared to 0crit group. Despite the lack of correlation between the IFL and addiction-like behaviors, NAc shell and amygdala were found correlated with resistance to punishment and/or persistence to seek cocaine. Interestingly, there was also correlation of Arc expression between brain regions, including cingulate cortex, OFC, NAc core and shell, AMY and DS, indicating that they may be a neuronal ensemble for cocaine seeking behavior. Dendritic spine density was not statistically significant, likely caused by the small group size.

A numerous of studies have demonstrated that chronic cocaine abusers show dysfunction of the frontal lobe, including impairments in delayed recall, deficits in attention or concentration tasks, increased perseverative errors on the Wisconsin card sorting task, which is indicative of deficits in inhibitory control (Beatty et al., 1995; Rosselli and Ardila, 1996). Human neuroimaging studies found correlation of those cognitive dysfunctions and hypofunction of the frontal area in cocaine abusers (Goldstein and Volkow, 2002). In preclinical studies, it has been demonstrated that Arc expression increases after systemic injections of a numerous of psychostimulants, including methamphetamine (Kodama et al., 1998; Yamagata et al., 2000), methylphenidate (Chase et al., 2007), amphetamine (Gonzalez-Nicolini and McGinty, 2002; Klebaur et al., 2002) and cocaine (Fosnaugh et al., 1995; Fumagalli et al., 2006; Zavala et al., 2008) in several brain regions. Assessments of Arc mRNA expression following cue-induced reinstatement of rats subjected to repeated cocaine self-administration showed increased mRNA levels in ACC, Prl, IFL, OFC, BLA, CPu and NAc core in the cocaine-treated group compared to control yoked-saline group, indicating that these brain regions are responsive for cocaine-associated cues (Zavala et al., 2008). Furthermore, Arc is a plasticity-associated gene of post-synaptic neurons as well as it is involved in cytoskeletal rearrangements (Fujimoto et al., 2004; Lyford et al., 1995), indicating that Arc expression in these brain regions might be relevant for functional long-term changes after administration of drugs of abuse (Robinson and Berridge, 2001; Robinson and Kolb, 1999, 2004). In our study assessing cocaine addicted-like and non-addicted-like rats, which show similarities in the genetic background, number of CSA sessions as well as similar number of cocaine infusions along life, but different addiction-like behaviors, we found significant lower expression of Arc in the IFL cortex in the 3crit compared to 0crit. It is unclear whether 3crit decreased the Arc expression in this brain region and/ or the 0crit increased the Arc expression. Nevertheless, the mPFC appears to play an important role in cocaine compulsive behavior, in agreement to human studies.

It has been previously demonstrated that dorsal mPFC (Cg and part of Prl) is involved in reward- and fear-related conditioning as well as it is essential for maintaining instrumental responding for appetitive stimuli, whereas the ventral mPFC (part of Prl and IFL) is associated with response inhibition in both aversive and appetitive domains (Gourley and Taylor, 2016). mPFC receives

input from amygdala, hippocampus and other limbic structures, integrating information of salience, value, and contextual cues associated with appetitive and aversive outcomes (Gabbott et al., 2006; Jay and Witter, 1991), indicating to be an important hub to guide behavior. Different to Prl cortex that is suggested to serve as a "go" structure and to produce energizing expression of reward-related behavior due to its large innervation with the NAc core, the IFL compartment guides the "stop" (Gourley and Taylor, 2016). Therefore, the decreased activity of the IFL cortex in 3crit during the cocaine-seeking behavior may indicate dysfunction of this brain region, which is in agreement with human studies. Previous studies in this animals model have demonstrated impairments of LTD in 3crit rats in the NAc core and Prl regions (Kasanetz et al., 2010; Kasanetz et al., 2013), brain regions that are critical for initiating cocaine seeking (Peters et al., 2008). Taken these studies together, it appears that the mPFC is dysfunctional in the cocaine addicted-like rats. Although speculative due to the lack of significance in our data, Arc expression in Prl and IFL regions appear to go in opposing directions and this may also suggest that the addiction behavior of 3crit rats may be strongly influenced by the Prl region.

Peters et al. (2008) have demonstrated that IFL and NAc shell are both necessary for extinction learning to inhibit cocaine seeking. Nevertheless, inactivation of these structures before extinction training did not affect cocaine-priming reinstatement (Peters et al., 2008). This result may indicate that both NAc shell and IFL are necessary for inhibiting the behavior during extinction training. IFL and NAc shell connects to each other by dense glutamatergic projections (Sesack et al., 1989), and in fact, although 3crit showed slower reduction in the number of nose-pokes during extinction training (data not shown) the 3crit group did reach the extinction criterion, suggesting that NAc shell may be (slowly) recruited to stop the behavior. Nevertheless, when facing the salience of cocaine or cocaine-associated cues, other neural networks may be activated and surpass the inhibitory response, leading to drug seeking behavior. For example, activation of Prl cortex is essential for cocaine-, cue-, and stress-induced reinstatement of extinguished cocaine self-administration (Capriles et al., 2003; Di Ciano and Everitt, 2004a; McFarland and Kalivas, 2001; McFarland et al., 2003), supporting our hypothesis mentioned above.

In our study, NAc shell positively correlated with both persistence of cocaine seeking and resistance to punishment (Fig. 15). NAc shell indeed appears to be essential for cocaine-seeking behavior. Infusions of amphetamine intra-NAc shell enhanced the motivational effects of conditioned stimuli on instrumental behavior (Wyvell and Berridge, 2000), while lesions of this brain region impaired the enhanced effects of cocaine- and d-amphetamine-associated stimuli (Ito et al., 2004; Parkinson et al., 1999). Although during both persistence of cocaine seeking and resistance to punishment tests the cue-lights are presented, in our experimental design is unclear whether the positive correlation between neuronal activity in NAc shell and instrumental response represents the salience of the conditioned stimuli that influence the instrumental response or it is a measure of cocaine-seeking behavior itself had we consider the salience for both groups are equivalent. Resistance to punishment also positively correlated with Arc expression in AMY. During the resistance to punishment test, foot-shocks are contingently delivered. AMY is involved in emotional states and it has been previously shown to be more active in cocaine abusers than healthy controls. The correlation in Arc expression was also found between brain regions. ACC was positively correlated with both OFC and NAc shell, whereas NAc shell correlated with DS. DS, on the other hand, correlated with NAc core, while NAc core correlated with AMY. Simultaneous activation of these brain regions during cue-induced reinstatement may indicate a neuronal ensemble for cocaine-seeking behavior. Nevertheless, IFL cortex was not found correlated with any brain regions, indicating that despite the activation of the mentioned neuronal ensemble, other brain regions are also playing a role in the compulsive behavior.

Dendritic spines density was also assessed in the mPFC of 0crit and 3crit rats. In our preliminary results, we found no significant difference between the groups. Nevertheless, this lack of difference could have been caused by small group size (n=2/ group). Additional studies to increase the group size are needed to confirm this result. Furthermore, the E-SARE expression was very dense in the mPFC, which produced difficulties to follow single dendrites for spines measurements. Other in vitro methodologies may be needed for dendritic analysis.

In conclusion, increased compulsive behavior for drug taking and seeking of addicted-like rats may be caused by the decreased neuronal activity in the infralimbic cortex. Nevertheless, further investigations are needed to clarify how the brain activity and neuronal functions of cocaine addicted-like and non-addicted-like rats changes over the course of drug use. In addition, further studies are needed to clarify the dendritic spine density in these groups.

Chapter 4: Pavlovian Instrumental transfer

Addiction is a complex and multifaceted mental disorder in which drug abusers loses the control over the drug taking and seeking behaviors. Understanding the underpinning mechanisms that lead to the pathological behavior is a challenge to the field and several psychological processes have been suggested to contribute to it. Among them, Pavlovian conditioning clearly plays a role. It is well known that conditioned stimuli (CS) trigger craving and relapse in drug abusers.

In recent decades the interaction processes of Pavlovian conditioning on influencing instrumental response have been widely studied and proposed to play a central role in addiction (Belin et al., 2009; Everitt et al., 2001; Everitt and Robbins, 2016). In instrumental conditioning, two general processes have been identified for positive reinforcement: the goal-directed action and the habit formation. During the development of addiction, it appears that the initial goal-driven action, which is motivated by reinforcement, is eventually transferred to a stimulus-response habit, which acquires autonomy and dominates the behavior. Habit is a behavioral adaptation that is outside of conscious awareness, but efficient to carry information and react rapidly when needed (Everitt and Robbins, 2016). During the development of addiction and due to much repetition of drug taking actions, habits eventually become maladaptive and persistent, producing the compulsive response displayed by drug addicted subjects (Dalley et al., 2011).

The Pavlovian-instrumental transfer (PIT) paradigm is a valuable tool to understand the motivational influence of Pavlovian conditioning on instrumental response. In this paradigm, subjects are trained in Pavlovian stimulus-outcome separated from instrumental response-outcome training to avoid associations between the cue and instrumental action. During the PIT test, the CS is non-contingently presented and the instrumental actions assessed throughout the test. Any increase in the instrumental performance during the CS presentation can signify the invigorating properties of the cue in the expression of instrumental behavior. Previous studies evaluating PIT effects of alcohol- or cocaine-paired conditioned stimuli have been inconsistent, showing both no-effect or increased CS-elicited responses (Lamb et al., 2016). In order to systematically investigate whether Pavlovian to instrumental transfer can predict individuals that

are vulnerable for the development of addictive behaviors, our aim was to use the translational 0/3crit animal model of cocaine addiction on the PIT paradigm.

Material and methods

Animals

Thirty-six eight-week-old male Sprague-Dawley rats from Charles River (Sulzfeld, Germany) were used for cocaine self-administration training. Animals were acclimatized for a week before catheter implantation. Following surgery, animals were housed individually in standard cages (Type-III; Ehret, Emmendingen, Germany) throughout the study. Temperature was controlled (22±2°C), and rats were maintained under reverse 12/12-hour light/dark cycle (lights on at 7:00 p.m.). Standard laboratory rat food (Sniff, Soest, Germany) was given 20g daily and drinking water was provided *ad libitum*, except when indicated. All experimental procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe), and carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs

Cocaine hydrochloride (Sigma-Aldrich, Taufkirchen, Germany) was dissolved in sterile saline (0.9% NaCl). Sucrose (Panreac AppliChem, Darmstadt, Germany) was dissolved in drinking water for a 10% solution.

Surgery

A polyurethane catheter (internal diameter: 0.58 mm, external diameter: 0.94 mm) was implanted in the jugular vein under isoflurane anesthesia (induction: 5%; maintenance: ~ 2.5%). The proximal end of the catheter was inserted in the right atrium of the animal´s heart, while the distal end was placed under the skin and fixed in the mid scapular region. Rats were allowed to recover for 4 to 7 days after the surgery. After recovery, catheters were flushed daily with saline solution containing unfractionated heparin (100 IU/ml) and Baytril® (1 mg/ml).

Operant cocaine self-administration apparatus

CSA trainings were carried out in nose-poke operant chambers (40 cm long x 30 cm width x 52 cm high; Imetronic, France) enclosed in ventilated sound-attenuating cubicles. Two nose-poke holes at opposite walls, 5 cm above the grid floor, recorded the responses by the interruption of a photo-beam in the hole. Poking in the active hole resulted in the delivery of an infusion of 0.8 mg/kg of cocaine, whereas poking in the inactive hole had no programmed consequences. The chambers were equipped with a white cue-light on the right hand-side placed 9.5 cm above the grid floor, a green cue-light placed next to the white-cue light, a blue cue-light located on the left hand-side 33 cm above the grid floor and a house light that illuminate the entire chamber. Data was recorded with POLY software.

Cocaine self-administration training

CSA protocol was performed as described in Vengeliene et al. (2018). Briefly, each CSA session consisted of alternated periods of drug availability (drug-ON, 40 min) and non-availability (NO-drug, 15 min). During drug-ON periods, a blue cue light signaled the availability of cocaine and a white-cue light (on for ~ 2s) was paired with every cocaine infusion (0.8 mg/kg/infusion). Cocaine was delivered under the fixed-ratio 5, e.g. five nose-pokes are required to deliver one cocaine infusion, which had to be completed within 40s. During NO-drug periods, blue and white cue-lights were withdrawn and a house light indicated the non-availability of cocaine. Nose-pokes were recorded, but cocaine was not delivered. Each cocaine infusion was followed by a 40 s time-out period and animals were trained for 2.5 h a day.

Following 45 CSA trainings, the three addiction behaviors were tested.

Motivation to self-administer the drug: Break point test was based on the progressive-ratio schedule of reinforcement. Drug availability was signaled by the blue cue light and the ratio of responses was increased after each cocaine infusion, according to the following progression: 10, 20, 30, 45, 65, 85, 115, 145, 185, 225, 275, 325, 385, 445, 515, 585, 665, 745, 835, 925, 1025, 1125, 1235, 1345, 1465, 1585. The test elapsed either after 6h or 1h from last completed ratio. The last completed ratio performed by the rat is referred as the breaking point.

Persistence to seek the drug: Persistence of cocaine-seeking was measured as the average number of active nose-pokes during NO-drug periods in the last four CSA training sessions prior to the BP test.

Resistance to punishment: The test was performed about 4 days following the BP test, and it consisted of FR5 schedule with cocaine infusion paired with foot shocks (0.2 mA, 1s) in a 40 min test. Additionally to the blue and white cue lights, a green cue-light was turned on after the first nose-poke to indicate the presence of shock. Two foot-shocks were delivered, the first shock was given at the fourth nose-poke, and the second shock was paired with cocaine infusion on the fifth nose-poke. Percentage of cocaine infusions earned during the test in comparison to baseline infusions during CSA training is used as a measurement of the criteria.

Addiction characterization: Rats showing responses above the 60th percentile of the population distribution in all three addiction criteria are classified as addicted-like (3crit), whereas below this threshold in all three assessments, as non-addicted-like rats (0crit). Seven 3crit and eight 0crit rats were produced and subjected to PIT paradigm.

Pavlovian instrumental transfer paradigm

The protocol was developed in our laboratory by Dr. Thomas Enkel, as follow:

Animals

Thirty two eight-weeks-old male Wistar rats from Harlan Laboratories (Derby, United Kingdom) were used for the validation of PIT protocol. Animals were housed individually in standard cages (Type-III; Ehret, Emmendingen, Germany) and acclimatized for a week before beginning of the conditioning. Temperature was controlled (22±2°C), and rats were maintained under reverse 12/12-hour light/dark cycle (lights on at 5:00 p.m.). Standard laboratory rat food (Sniff, Soest, Germany) and drinking water were provided *ad libitum*, except when indicated. All experimental procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe), and carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Operant self-administration apparatus for Pavlovian instrumental transfer

Pavlovian instrumental transfer paradigm was carried out in lever-presses operant chambers (MED Associates Inc., St. Albans, VT) enclosed in ventilated sound-attenuating cubicles. The chambers were equipped with two levers placed at opposite walls. Responses at the active lever activated a syringe pump that delivered a ~30µl drop of sucrose into a liquid receptacle that was next to it. Responses at the inactive lever were recorded, but had no programmed consequences. A light stimulus is attached above both response levers of the operant chamber. Delivery of sucrose, presentation of stimuli and data recording was controlled by a computer equipped with MED-PC software (MED Associates).

Habituation: On the day before the habituation in the operant chambers, animals were water-deprived for 22h. Habituation lasted 1h and, during this time, sucrose was delivered in variable interval (VI) of 120s, but levers were not present.

Pavlovian conditioning: Pavlovian conditioning took place for 60min for five consecutive days. A conditioned stimulus (CS) – cue-light – was presented for 120s and paired with two drops of sucrose (1 reward). For each CS presentation, 4-5 rewards were given in an inter-trial interval of 120-360s. A total of 9 pairings of CS and rewards were given per session. Control group received 2.5 ml of sucrose in the delivery well prior to start the session to avoid association between the CS and reward. Levers were absent throughout the Pavlovian session.

Instrumental training: Instrumental training started on the next day after the end of Pavlovian conditioning and 22h prior to instrumental conditioning animals were water-deprived. In this phase, cue-lights were withdrawn and rewards were delivered by pressing the active (right) lever. Each session lasted 30min and it was carried out in progressive variable intervals (VI) for the delivery of reward. The first three days animals were trained on a fixed ratio 1 (FR1) for acquisition of instrumental response. On the subsequent ten days, the average VI was as follow: 1 day VI-10 (5s, 10s, 15s), 1 day VI-20 (10s, 20s, 30s) and 8 days VI-30 (10s, 20s, 30s).

Reminder: A single 30 min session of Pavlovian training was given on the day before the PIT test. The conditions were the same as the Pavlovian training, as described above.

PIT test: The transfer test took place on the day after the reminder session. Both active and inactive levers were constantly available, however rewards were not delivered during the test. Cue-light was presented for 120s in intertrials of 10, 8, 6 and 4 min. In total, there were four cue-light presentations in a 36min test. Lever presses were recorded throughout the PIT test.
Number of lever presses prior (2 min) and during (2 min) the CS presentation was used to calculate the transfer effect. Total lever presses in both active and inactive lever were also considered to assess the performance.

Data analysis
For comparison between addicted-like (3crit) and non-addicted-like (0crit) rats in addiction-like behaviors, or the PIT transfer (%), Student t-test analysis was used. Data obtained from number of lever presses during PIT test (pre-CS vs. CS) or performance in instrumental training, repeated measures analysis of variance (ANOVA) was applied. Pearson Correlation was used to assess linear relationship between performance in the CSA and transfer in PIT. Whenever significant differences were found, Bonferroni post-hoc test was performed. The chosen level of significance was $P < 0.05$. Data were analyzed using Statistica and GraphPad Prism software.

Results
Validation of Pavlovian to instrumental transfer paradigm in our laboratory conditions
PIT validation was performed by Dr. Valentina Vengeliene and Sabrina Koch.

For validation of the Pavlovian to instrumental transfer paradigm in our laboratory conditions, animals were subjected to Pavlovian and instrumental conditioning or to instrumental training only (controls). Control rats were placed in the operant chambers during the Pavlovian phase; however all the corresponding rewards were given at the beginning of the session to avoid associations between CS and reward delivery. Our results demonstrate that PIT group showed increased number of lever presses during the presentation of CS in comparison to pre-CS period [Fig. 25-A, pre-CS vs CS: $F_{(7, 210)} = 16.08$, $P < 0.0001$; groups: $F_{(1, 30)} = 33.26$, $P < 0.0001$; interaction: $F_{(7, 210)} = 7.12$, $P < 0.0001$]. Control group showed no difference in the number of

lever presses between pre-CS and CS period (P>0.05). PIT group showed transfer of the Pavlovian to instrumental response of approximately 74%, whereas control group showed 48% of lever presses during CS presentation (Fig. 25-B), indicating that CS did not produce any influence in the instrumental response in the control group.

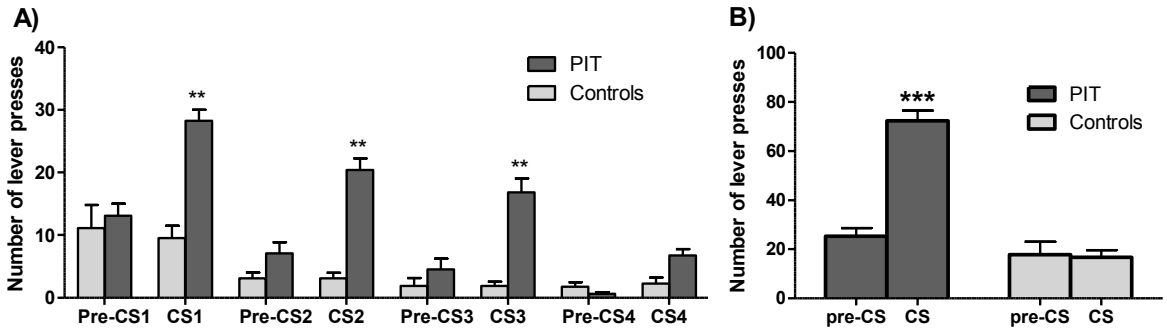


Figure 25 Pavlovian to instrumental transfer test for validation of the protocol
Transfer test was performed following Pavlovian and instrumental trainings. Control group received instrumental conditioning only; PIT group received Pavlovian and instrumental conditioning. Data is shown as mean ± SEM of the number of active lever presses pre-CS and during CS presentation in the PIT test (A), and total number of lever presses in pre-CS and CS periods during the test (B). **P<0.001 and *** P<0.0001 compared to pre-CS.

Characterization of addiction-like behavior

Following about 45-50 CSA training sessions, all animals were subjected to addiction-like behavior tests. Scores of 0 or 1 was given to each animal showing higher or lower than 60th percentile of the population distribution in each addiction criteria test. Out of the 36 rats that successfully completed the CSA training, seven rats showed positive (3crit) for all tested criteria, whereas eleven rats showed negative for all tested criteria (0crit). Seven addicted-like (3crit) and eight non-addicted-like (0crit) rats were subjected to PIT paradigm. Student t-test showed significant difference between performance of 3crit and 0crit in each addiction criteria: motivation [t(13) = 7; P<0.0001, Fig. 26-A], persistence of drug-seeking [t(13) = 2.8; P=0.014, Fig.26-B] and resistance to punishment [t(13) = 5.3, P=0.0001, Fig. 26-C].

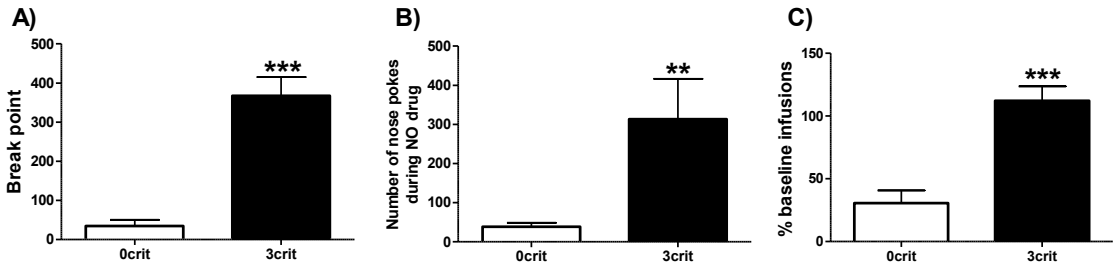


Figure 26 Characterization of Addiction-like behaviors.
Bars represent mean ± SEM for each of the addiction-like behaviors of non-addicted-like (0crit) and addicted-like (3crit) (n = 7-8/ group). ** P=0.014; *** P≤ 0.001.

Transfer from Pavlovian to instrumental response were similar between addicted-like and non-addicted-like rats

At the end of the CSA training (~55 sessions), animals were undisturbed for 1-2 weeks prior to the Pavlovian to instrumental transfer paradigm. Both 0crit and 3crit showed equivalent acquisition of the instrumental learning [Fig. 27-A-D, groups: P = 0.21; interaction: P = 0.69]. In the last five instrumental training sessions, 0crit group showed 265±34 active lever presses and 3crit showed 360±70 active lever presses. Interestingly, although 3crit showed slightly higher number of active lever pressing compared to 0crit in the last instrumental training (P = 0.22), the number of inactive lever presses and number of rewards earned were not different from 0crit animals. Transfer effects were also akin between the groups [Fig. 27-E, F, time: $F_{(7, 140)} = 28.04$, P<0.0001; groups: $F_{(2, 20)} = 0.44$, P = 0.65; interaction: P = 1.0]. Transfer of Pavlovian to instrumental response in 0crit corresponded to about 77 %, while 3crit about 78 % (Fig. 27-F).

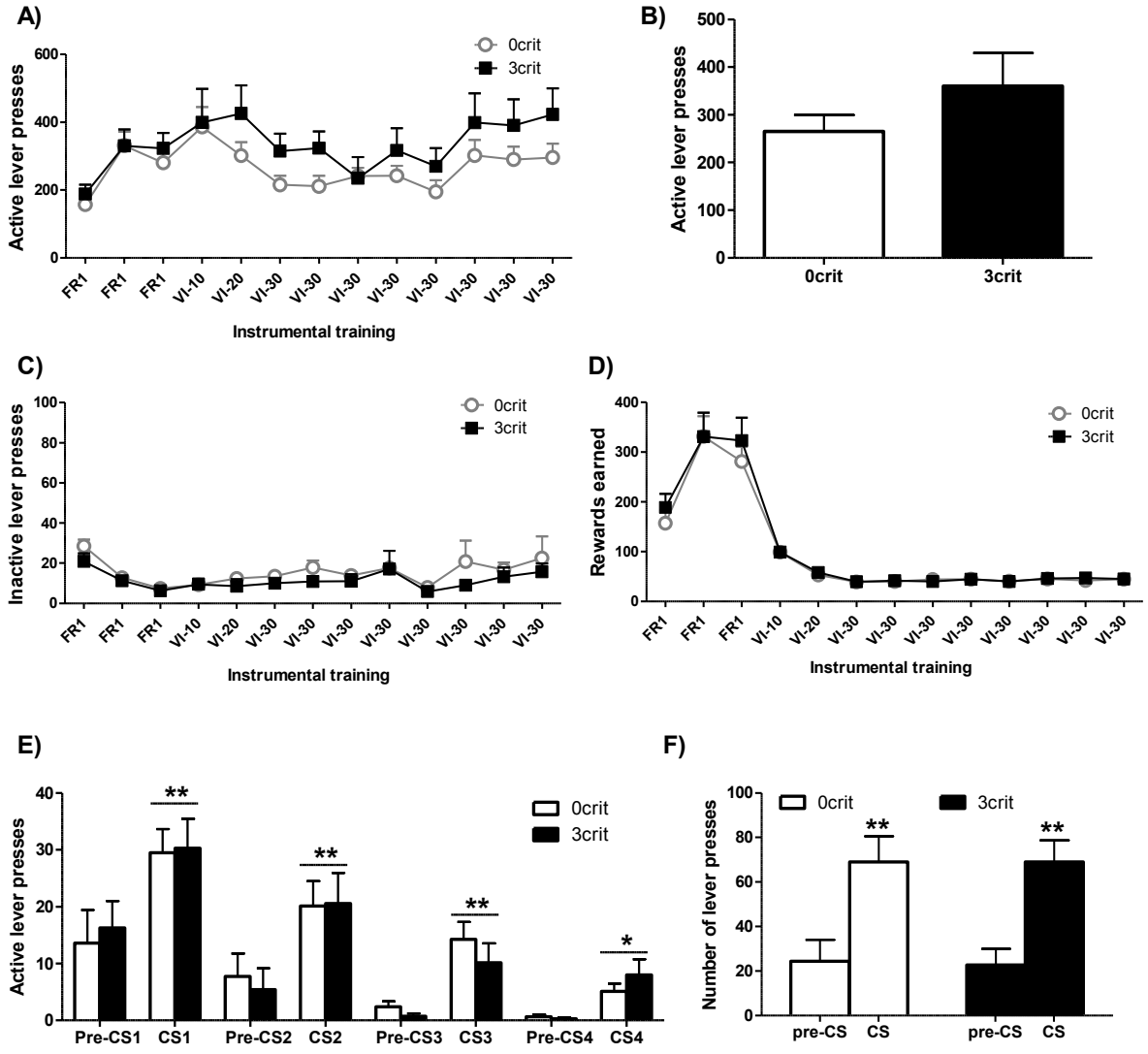


Figure 27. Performance of addicted-like (3crit) and non-addicted-like (0crit) rats in the Pavlovian to instrumental training and test.

Instrumental response between 0crit and 3crit groups neither differed in the numbers of active (A) and inactive (C) lever presses nor in the number of rewards earned (D). Active lever presses in the last five instrumental sessions (VI-30) were also similar between groups (B). PIT test was similar in both 0crit and 3crit groups during pre-CS and CS presentations (E) and in total number of lever presses in each period (F). **P<0.001 compared to pre-CS. Data is shown as mean ± SEM.

Performance in the cocaine self-administration training correlates with transfer from Pavlovian to instrumental response

There was a positive correlation between the performance in the last five CSA sessions (%) and the transfer in the Pavlovian to instrumental response [Fig. 28, r = 0.57; n = 15; P = 0.025]. Performance in the CSA was calculated according to the following equation: [(number of nose pokes in the active hole/ number of total nose pokes)*100]. Addiction behaviors were also analyzed; however, no correlation was found with performance in PIT [PIT vs motivation: r = 0.44; P = 0.39; persistence: r = -0.35, P = 0.49; resistance to punishment: -0.16; P = 0.76].

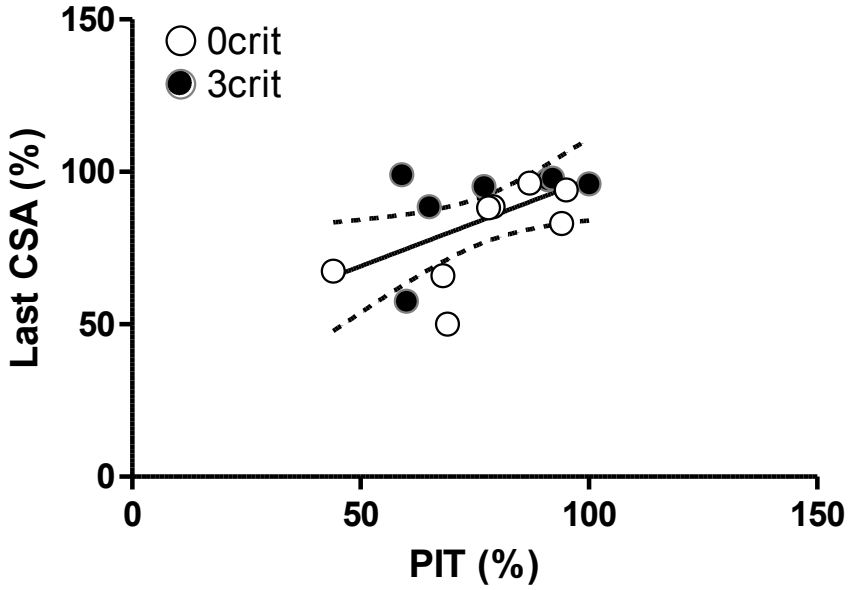


Figure 28. Pearson correlation between performances in the last CSA training sessions of 0/3crit and transfer of Pavlovian to instrumental response.
Positive correlation between performance in the last five CSA sessions and PIT (r = 0.57; n = 15; P = 0.026).

To assess whether this correlation is robust and results were not affected by the pre-training in the operant paradigm with cocaine, a new group of naïve rats were subjected to PIT paradigm and thereafter to CSA training. All addiction-like behaviors were tested between CSA sessions 22 – 27, and results of both PIT and CSA were used for Pearson's correlation analysis. Similar to the

first results, we have found a positive correlation between performances in the last five CSA training sessions (~33 sessions) and transfer in the Pavlovian to instrumental response [Fig. 29, r = 0.61; n = 20; P = 0.0046]. In addition, none of the addiction behaviors either correlated in this group [motivation: r = -0.21; P = 0.46; persistence: r = 0.19; P = 0.49; resistance to punishment: r = 0.21; P = 0.46].

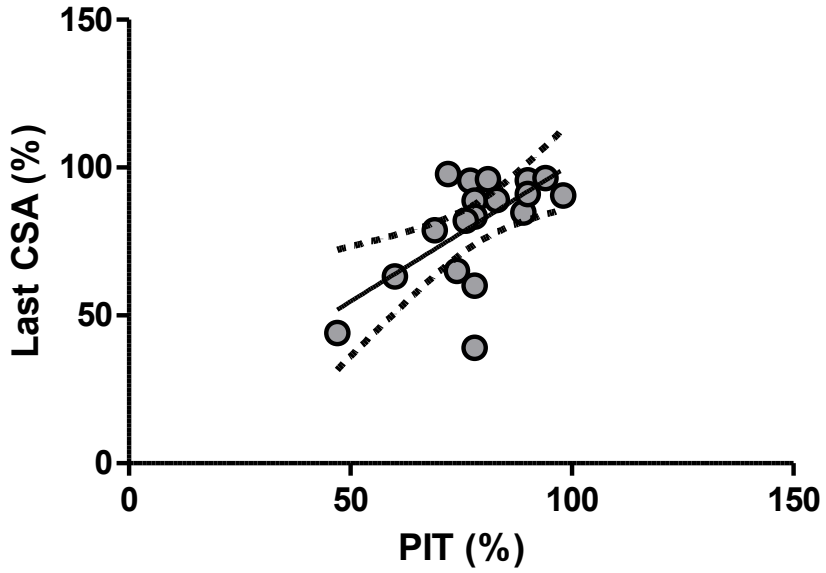


Figure 29. Pearson correlation between response in the Pavlovian to instrumental transfer test and the performance in the last five CSA training sessions.
Animals were first subject to PIT paradigm and thereafter to CSA training. Correlation analysis between performance in PIT and last CSA sessions showed positive correlation (r = 0.61; n = 20; P = 0.0046).

Discussion

The present study demonstrates that substance use severity did not correlate with transfer in the Pavlovian to instrumental paradigm. However, there was a robust correlation between performances in the last CSA sessions and the transfer (%) in PIT, suggesting that Pavlovian to instrumental transfer may predict drug-taking behavior rather than predict the risk for addiction.

Our protocol validation showed that the control group, which received only instrumental conditioning, did not show any transfer during the test, as expected, indicating that transfer of

animals subjected to both Pavlovian and instrumental training is not coincidental. The Pavlovian to instrumental transfer paradigm has been used to understand the motivational influences on instrumental performance and it has been suggested to predict substance use disorder (Garbusow et al., 2016; Garbusow et al., 2014; Heinz et al., 2017). For instance, Garbusow et al. (2016) found positive correlation between monetary and drug-related cues in alcoholics tested in PIT paradigm. However, in our controlled experimental design the cocaine addicted-like (3crit) and non-addicted-like (0crit) rats neither differed in the acquisition of instrumental learning nor in the transfer of the Pavlovian to instrumental response. This discrepancy between our data and human data may be caused by different testing groups. Different to the human studies that compared alcoholics vs. healthy controls, we have compared addicted-like and non-addicted-like rats, which had similar training days in operant conditioning for long-term cocaine self-administration, and the animals not only show similar genetic background, history of drug use and environmental conditions, but also comparable number of cocaine infusions. Although one may think that the previous operant conditioning experience could facilitate the performance in PIT paradigm, the naïve groups that were first tested in PIT and thereafter in cocaine self-administration also showed no correlation between performance in PIT and addiction-like behaviors. However, we found a positive correlation between performance in PIT and in CSA, suggesting that PIT may rather predict the drug taking behavior.

The similarities in the acquisition of instrumental training of 0crit and 3crit also suggest that learning capacity of an operant response might be akin between groups. Interestingly, the 3crit group showed a tendency for increased number of active lever presses during the later phase of instrumental training, without changing in the number of inactive lever presses. Further investigations are needed to clarify that; however two possibilities could be thought to explain this result: (1) addicted-like rats may be more persistent to seek reward, either as a pre-existing feature or as a result of carry over effect from previous conditioning, or (2) addicted-like rats may rapidly develop habitual behavior to seek the reward, which would be more under the control of "automatized" response. More studies are warranted to answer this hypothesis.

One of the limitations of our study is the inability to evaluate the motivational properties (salience) of the Pavlovian-CS in addicted-like and non-addicted-like rats and the influence of other etiological factors on PIT performance. It has been shown that transfer of Pavlovian to instrumental response may be influenced by stress. Some clinical studies failed to find PIT effects in all drug dependent subjects and suggested that impulsive choice behavior or stress may play as an important factor for higher transfer (Dias-Ferreira et al., 2009; Morgado et al., 2012; Pielock et al., 2013; Quail et al., 2017a; Quail et al., 2017b; Schwabe et al., 2011a; Schwabe et al., 2011b; Schwabe and Wolf, 2010). In our experiment, both addicted-like and non-addicted-like rats were tested in PIT during protracted abstinence to cocaine (PIT training and test were performed during 5-6 weeks of cocaine abstinence). Although withdrawal effects were not assessed in our animals, both 0crit and 3crit groups showed similar transfer to each other, 77 % and 78 %, respectively, which were 3-4% higher than the drug-naïve control rats tested for PIT protocol validation.

Another limitation of the present study is the inability to distinguish between specific or general transfer as we only used a single CS and a single lever paired with sucrose in PIT. Considering that 0crit and 3crit show significantly different addiction-like behaviors to cocaine, a specific transfer test could provide better insights about transfer in 0crit and 3crit rats to cocaine. Nevertheless, following training in the PIT paradigm, we have retrained the animals in the CSA and tested for cue-elicited cocaine seeking (data not shown). Performance of both 0crit and 3crit in the CSA and cue-induced reinstatement test were not affected by PIT conditioning. The 3crit group showed increased number of active nose-poke compared to 0crit in both assessments as typically found in this animal model.

In conclusion, PIT paradigm appears to be a useful test to predict drug taking behavior. However, it appears to be insufficient to predict severity of substance use disorder and other factors than PIT effects might be involved and playing a greater role in defining the risk to develop addiction.

Chapter 5: Novel medications to treat cocaine addiction

To date, there are no commercially available medications to help psychostimulant abusers to prevent relapse, reduce drug craving, and/or attenuate physiological dysfunctions, such as those produced by withdrawal effects. The high failure rates in the Phase III and IV of clinical trials could be related to preclinical studies performed in animals subjected to acute or sub chronic drug treatments. Although there is no animal model that can fully mimic the human addiction, they are helpful to study specific features of the disease (Koob and Nestler, 1997). Nevertheless, changes in the brain network and response to drugs following chronic and acute drug treatments are certainly different. Therefore, using translational animal models to assess novel drugs are essential.

Drug addiction produces dysfunction of several neuronal systems as introduced in the chapter 1 of this thesis. Hence, one of the major challenges in pharmacotherapy is to specifically target a receptor or physiological system that is affected or deranged by drug of abuse (Kreek et al., 2002). A common strategy for testing novel drugs is targeting a very specific receptor in the disrupted neurotransmitter systems. This strategy decreases the amount of side effects. Usual targets, such as glutamate receptor antagonists and $GABA_B$ agonist baclofen, have been tested elsewhere and failed in the clinical trials or pharmacovigilance reports showed concerns over the utilization of the drug (ANSM, 2017). Here, different approaches were used. A novel $GABA_B$ positive allosteric modulator CMPPE was tested in comparison to baclofen in order to produce lesser side effects; melatonin was tested for regulation of circadian rhythm; and specific NMDAR subunit was target for attenuation of addiction behaviors as an alternative to NMDAR antagonists.

5.1 GABAergic system: positive allosteric modulator of $GABA_B$ receptor

This study was performed in collaboration with Dr. Valentina Vengeliene on alcohol addiction (Vengeliene, Takahashi et al., Psychopharmacology, 2018). However, here I will show only the results on cocaine-seeking behavior.

Introduction

$GABA_B$ receptor is a heterodimer of two heptameric membrane receptor subunits, the $GABA_{B1}$ and $GABA_{B2}$ subunits (Jones et al., 1998; White et al., 1998). The receptor is a member of the G-protein coupled receptor family and is found both pre-and post-synaptically (Kulik et al., 2006; Kulik et al., 2003; Ladera et al., 2008; Lopez-Bendito et al., 2004; Lujan and Shigemoto, 2006; Ulrich and Bettler, 2007; Vigot et al., 2006). Its post-synaptic effects are exerted by activating the G-protein coupled inwardly rectifying potassium (GIRK) channels, inducing slow inhibitory potential (Gahwiler and Brown, 1985; Newberry and Nicoll, 1984; Sodickson and Bean, 1998). Pre-synaptically, $GABA_B$ receptors function as inhibitory auto- and heteroreceptors in both GABAergic and glutamatergic nerve terminals (Brager et al., 2003; Dittman and Regehr, 1996; Isaacson and Hille, 1997; Sakaba and Neher, 2003; Scanziani et al., 1992; Takahashi et al., 1998; Vigot et al., 2006), either inhibiting Ca^{2+} channels and thus reducing neurotransmitter release (Perkinton and Sihra, 1998; Takahashi et al., 1998; Tareilus et al., 1994) or through modulation of Ca^{2+} influx downstream process (Dittman and Regehr, 1996; Sakaba and Neher, 2003; Scanziani et al., 1992).

In recent years, $GABA_B$ receptor agonist baclofen has been prescribed for the treatment of alcoholism in the clinic. This was likely influenced by several preclinical studies performed in the last decades, which have demonstrated the efficacy of baclofen in reducing both voluntary alcohol drinking and the motivation to self-administer alcohol, as well as effective to abolish the relapse-like alcohol drinking behavior (Colombo et al., 2002; Colombo et al., 2003a; Colombo et al., 2006; Colombo et al., 2003b; Maccioni et al., 2012). Baclofen was also effective to reduce cocaine cue-elicited reinstatement and the acute rewarding effects of cocaine (Brebner et al., 1999; Di Ciano and Everitt, 2003; Roberts et al., 1996; Slattery et al., 2005; Xi and Stein, 1999). However, in the above mentioned preclinical studies baclofen showed no apparent side effects, such as sedation or body weight loss (Agabio and Colombo, 2014). Conversely in the clinic, alcohol addicted patients have reported several undesirable effects by the administration of the $GABA_B$ receptor agonist baclofen, including substantial fatigue and sleepiness (Kiel et al., 2015; Pelissier et al., 2017). Furthermore, in July 2017 the French National Drug and Health Products Safety Agency released a warning about the increased hospitalization and premature deaths with

the use of baclofen for the treatment of alcoholism (ANSM, 2017). This discrepancy between clinical and preclinical studies may likely be caused by tests performed in non-dependent animals, which have had brief instrumental training procedures and assessments of acute or sub-chronic treatments of baclofen. These short experimental conditions might have contributed to the lack of side effects in preclinical studies. Considering the strong undesirable effects of baclofen in the clinic, an alternative to overcome this issue is to use positive allosteric modulators (PAM) of $GABA_B$ receptors. PAMs of $GABA_B$ receptors have been developed in the recent years showing less adverse side effects, once it only acts in terminals where GABA is already present. Because each PAMs show slightly different molecular mechanisms (Perdona et al., 2011), the aim of this study was to assess the effects of a novel positive allosteric modulator of $GABA_B$ receptor CMPPE (2-{1-[2-(4-chlorophenyl)-5-methylpyrazolo[1,5-a]pyrimidin-7-yl]-2-piperidinyl}ethanol) in cocaine-addicted rats and CMPPE effects with baclofen effects.

Material and Methods

Animals and cocaine self-administration

Animals were trained in the 03crit model of cocaine addiction. In the present study, only addicted-like (3crit) rats were used (n=12) for pharmacological assessments.

Drugs

Cocaine hydrochloride (Sigma Aldrich, Taufkirchen, Germany) was dissolved in sterile saline. Baclofen (Sigma-Aldrich, Germany or generously provided by AbbVie, Ludwigshafen, Germany) was dissolved in either saline or polyethylene glycol (PEG, Sigma-Aldrich, Germany) and then diluted with injection water to a final PEG concentration of 10%. CMPPE (generously provided by AbbVie, Ludwigshafen, Germany) was either suspended in 1% hydroxypropyl methylcellulose or dissolved in cremophor (Sigma-Aldrich, Germany) and then diluted with injection water to a final cremophor concentration of 20%. All drugs were freshly prepared and injected intraperitoneally (i.p.) in a volume of 2 ml/kg. The doses of baclofen and CMPPE and the timing of injections were selected according to the previous published research (Brown et al., 2016; Perdona et al., 2011).

Extinction phase

Following ~ 60 CSA training sessions, 3crit rats underwent extinction training. During the extinction sessions cue-lights and cocaine infusion were withdrawn. However, nose-poking in both active and inactive levers were recorded throughout the 2h daily session. After 9 days of training, animals reached the extinction criteria (<20% AP from baseline).

Cocaine cue-induced reinstatement

Reinstatement was performed on the next day after the final extinction session. In the reinstatement test, rats were exposed to the same conditions, blue cue-light, signaling drug availability, and contingent white cue-light, at the completion of FR5, were present, however cocaine was not available. The number of nose-poking in both active and inactive holes was recorded throughout the 1h test.

To test the effects of CMPPE and baclofen on the cue-induced reinstatement of cocaine- seeking, animals were divided into groups on the basis of their performance during the last CSA and extinction sessions (n = 7–8/ treatment group). Vehicle, 3 mg/kg of baclofen or 30 mg/kg of CMPPE was administered 30 min before the reinstatement test. Animals were subjected to this test once per week using a within-subjects Latin Square design.

Statistical analysis

Cocaine-seeking behavior was analyzed by a three-way ANOVA with repeated measures [factors were treatment, session (extinction vs. reinstatement), and nose-pokes (active vs. inactive)]. Whenever significance was indicated by ANOVA, Student Newman-Keuls' post-hoc tests were performed. The chosen level of significance was $P < 0.05$.

Results

Effects of the administration of baclofen and CMPPE on cue-induced reinstatement of cocaine-seeking behavior

At the end of the conditioning phase, 12 addicted-like (3crit) rats exhibited 477 ± 56 of active nose-pokes and 53±19 inactive nose-pokes. The number of operant responses progressively faded away across nine extinction sessions, reaching 39 ± 9 active nose-pokes and 30 ± 5 inactive nose

pokes. Cue-induced reinstatement was carried out on the next day after the extinction criteria was achieved. ANOVA for baclofen vs. vehicle treatment revealed significant difference between extinction and reinstatement sessions [factor session: $F_{(1,24)} = 7.5$, $P < 0.01$] and significant effect of baclofen treatment [factor session vs. treatment interaction $F_{(1,24)} = 7.1$, $P < 0.01$, and factors session vs. treatment vs. nose-poke interaction $F_{(1,24)} = 6.5$, $P < 0.05$]. Post-hoc analysis indicated that baclofen treatment abolished cue-induced cocaine-seeking behavior (Fig. 30-A). Similarly, analysis of vehicle vs. CMPPE data showed significant difference between extinction and reinstatement sessions [$F_{(1,26)} = 5.9$, $P < 0.05$]. Similar to baclofen effects, administration of 30 mg/kg of CMPPE also abolished cue-induced cocaine-seeking [factor session vs. treatment interaction $F_{(1,26)} = 5.0$, $P < 0.05$, and factor session vs. treatment vs. nose-poke interaction $F_{(1,26)} = 4.3$, $P < 0.05$] (Fig. 30-B). Post-hoc analysis indicated that nose-pokes in the inactive-hole were not significantly different in both baclofen- and CMPPE-treated groups (Fig. 30 A, B).

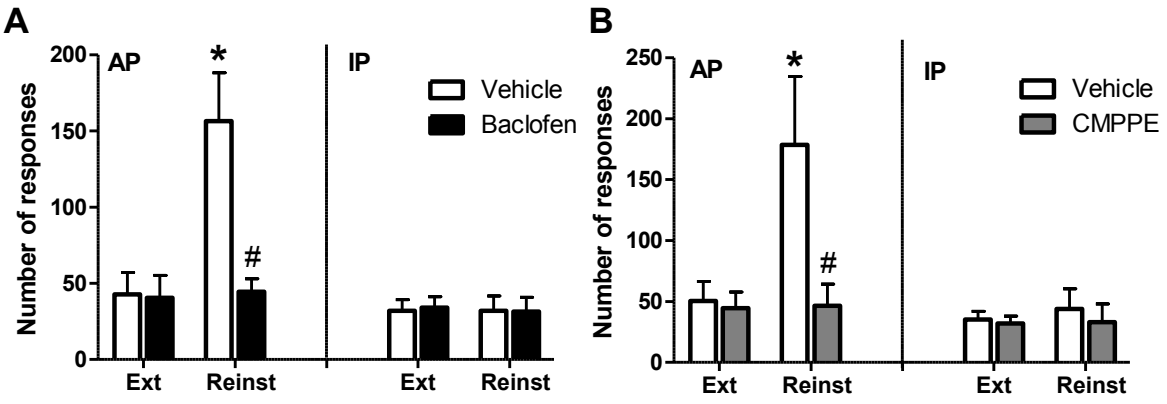


Figure 30 Baclofen or CMPPE effects on the cue-induced reinstatement test in cocaine-addicted rats.

The effects of either vehicle or 3 mg/kg of baclofen (A) and vehicle or 30 mg/kg of CMPPE (B) (n = 7–8/ treatment condition) on cocaine cue-induced reinstatement. Data is shown as the means ± S.E.M. of number of active (AP) and inactive (IP) nose-pokes during the last extinction sessions (Ext) and reinstatement test (Reinst). *Significantly different from extinction nose-pokes; #significantly different from the vehicle group ($P < 0.05$).

Discussion

The present study demonstrates that both baclofen and CMPPE produced abolishment of cocaine-seeking behavior in cocaine addicted-like rats. In cocaine-seeking assessment, both drugs showed no apparent side effects. However, the highest dose of the $GABA_B$ receptor agonist baclofen caused sedation and significant loss of body weight in alcohol addicted-like rats. These side effects were not observed in CMPPE-treated rats (Vengeliene et al., 2018).

Clinical studies have consistently reported systemic side effects of baclofen. Similarly, baclofen administration in alcohol addicted-like rats produced substantial undesired effects including reduction of locomotor activity, measured as reduced home-cage activity, and significant loss of body weight (Vengeliene et al., 2018). This result emphasizes the importance of using a translational animal model for pharmacological assessments.

Studies have demonstrated that CMPPE is able to stimulate [35S]GTPγS binding to membranes overexpressing $GABA_B$ receptors in similar level as the full agonist baclofen, and this stimulation was also higher than other PAMs, including CGP7930 and GS39783 (Perdona et al., 2011). This suggests that CMPPE shows efficacy comparable to baclofen and higher than other PAMs. However, akin to other PAMs CMPPE does not provoke development of pharmacological tolerance neither produce substantial side effects (Gjoni and Urwyler, 2008; Lehmann et al., 2003). Furthermore, activation of $GABA_B$ receptors appears to be effective to attenuate cue-induced cocaine seeking. Our data agrees with other studies performed in animals subjected to short-term alcohol or cocaine self-administration protocols (Agabio and Colombo, 2014; Colombo et al., 2003b; Filip and Frankowska, 2007; Filip et al., 2015; Froger-Colleaux and Castagne, 2016). Clinical data on cocaine addicted subjects also agrees with effectiveness of $GABA_B$ compounds on preventing relapse. For instance, Young et al. (2014) demonstrated that cocaine cue-induced neuronal activation of the ventral striatum, ventral pallidum, amygdala, and orbitofrontal cortex measured in cocaine-dependent participants were prevented by baclofen treatment. Taken these results together, targeting $GABA_B$ receptors may be effective in reducing cocaine-seeking responses in both short-term and long-term cocaine use.

5.2 Melatonin

Introduction

Melatonin is a pineal hormone that regulates circadian rhythm in mammals. Its secretion is controlled by the suprachiasmatic nucleus (SCN), and it rises during the dark phase of the light/dark cycle (Reiter et al., 1980). Melatonin synthesis and release precisely follow the environmental photoperiod, giving information of daily and annual time to the entire body (Castillo-Romero et al., 1993). Fluctuation of serum melatonin also controls important physiological functions, such as vascular tension, sleep-wake cycles, and reproduction (Hardeland et al., 2011; Lynch et al., 1975a).

Melatonin is synthesized from tryptophan to serotonin and is immediately secreted following synthesis. It binds to two melatonin receptors - MT_1 and MT_2 . Both subtypes are coupled to G-proteins that mediate inhibition of adenylyl cyclase (von Gall et al., 2002) and are found in brain areas relevant to drug effects and drug addiction, such as PFC, AMY, dorsal and ventral striatum, and hippocampus (Noori, 2012; Uz et al., 2005b).

Drugs of abuse have the ability to entrain the body clock (Shibata et al., 2010). Previous studies have shown that depending on the time-of-day that experiment is performed, drug sensitization, consumption and conditioned place preference differ (Akhisaroglu et al., 2004; Brick et al., 1984; Garmabi et al., 2016; Kurtuncu et al., 2004). Furthermore, drug abusers and addicted subjects display disrupted circadian rhythm as well as sleep-wake cycle, suggesting that melatonergic system may be playing a role in drug addiction (Abarca et al., 2002; Conroy et al., 2012; Drummond et al., 1998; Kovanen et al., 2010; Kuhlwein et al., 2003; McClung, 2007; Morgan et al., 2006; Peres et al., 2011; Spanagel et al., 2005a; Stowie et al., 2015). In fact, an earlier study from Spanagel's group have demonstrated that administration of melatonin or a non-selective melatonergic receptor agonist agomelatine in rats abolished relapse-like ethanol consumption and produced circadian phase advance, restoring the circadian rhythm (Vengeliene et al., 2015). Based on this, we have divided the study in two parts: (I) to investigate whether melatonin administration attenuates cocaine-seeking behavior as well as lower the motivation to self-

administer cocaine; (II) to investigate whether melatonin effects on cocaine-seeking behavior is mediated by MT_1 or MT_2 subtypes.

Material and methods

Animals

Two-month-old male Sprague-Dawley rats from Charles River (Sulzfeld, Germany) were used. Animals were acclimatized for a week before implantation of a catheter in the jugular vein. After surgery, the animals were housed individually in standard cages (Type-III; Ehret, Emmendingen, Germany) throughout the study. Temperature was controlled (22 ± 1 °C), and rats were maintained under reverse 12/12-h light/dark cycle (lights on at 7:00 p.m.). Standard laboratory rat food (Sniff, Soest, Germany) and drinking water were provided *ad libitum*. All experimental procedures were approved by the Committee on Animal Care and Use (Regierungspräsidium Karlsruhe), and carried out in accordance with the local Animal Welfare Act and the European Communities Council Directive of 24 November 1986 (86/609/EEC).

Drugs

Cocaine hydrochloride (Caelo, Hilden, Germany) was dissolved in water for injection. Melatonin (Sigma-Aldrich, Taufkirchen, Germany) was suspended in 0.5% methylcellulose (Sigma-Aldrich, Taufkirchen, Germany) and injected at a volume of 3 ml/kg intraperitoneally (i.p.). The control group received an equal volume of vehicle. Sucrose (Panreac AppliChem, Darmstadt, Germany) was dissolved in drinking water for 0.5% solution.

MT_2 antagonist 4-Phenyl-2-propionamidotetralin (4P-PDOT) was dissolved in 80% DMSO/saline and injected at a volume of 2 ml/kg. The MT_2 partial agonist (N-[(4-methoxy-1H-indol-2-yl)methyl]propanamide (UCM 924) was dissolved in 70% DMSO/saline and injected at a volume of 0.6 ml/kg. Both drugs were generously provided by Dr. Stefano Comai (San Raffaele Scientific Institute and Vita-Salute University, Italy).

Surgery

A polyurethane catheter (internal diameter: 0.58 mm, external diameter: 0.94 mm) was implanted in the jugular vein under isoflurane anesthesia. The proximal end was inserted in the right atrium

of the animal's heart, while the distal end was passed underneath the skin and fixed in the mid-scapular region. Rats were allowed to recover for 4 to 6 days after the surgery, receiving antibiotic treatment (enrofloxacin, Baytril® in the drinking water) for a week. After recovery, catheters were flushed daily with saline solution containing unfractionated heparin (100 IU/ml) and Baytril® (1 mg/ml).

Intravenous cocaine self-administration

All experimental procedures were carried out in operant chambers (Imetronic, France) enclosed in ventilated sound-attenuating cubicles. The cocaine self-administration (SA) protocol was based on Deroche-Gamonet et al. (2004) and Cannella et al. (2013). Briefly, the animals were trained during their dark phase for 70 CSA sessions in the operant chambers (40 cm long × 30 cm width × 52 cm high). Two holes, located at opposite walls at 5 cm from the grid floor, recorded the nose-poke responding. The implanted catheter was connected to a pump-driven syringe (infusion speed 20 µl/s). Animals were trained under fixed ratio 5 (FR5), and received an infusion of cocaine (0.8 mg/kg) paired with presentation of a white cue light (1.8 cm in diameter) located 9.5 cm above the active hole. Rats had access to a maximum 35 infusions per session to avoid overdose. Nose pokes at the inactive hole were recorded, but had no programmed consequences. Each CSA session comprised of alternated 40 min 'Drug-ON' period, signaled by a blue cue-light indicating drug availability, and 15 min 'No-drug' period, signaled by a house-light illumination. Nose pokes were recorded throughout the 2.5 h session. Data was collected with SK_AA software (Imetronic Company, France).

Experimental design

Melatonin effects were assessed on: (1) the motivation to work for cocaine in the break point test, the relapse-like behavior in the cue-induced reinstatement test, (3) the locomotor activity in the open field test, and (4) the sucrose preference in a two-bottle choice paradigm. Melatonin was administered 3–4 h after the onset of the dark phase, 30 min prior to each test, and the doses (25 and 50 mg/kg) were chosen based on our previous work (Vengeliene et al. 2015).

Break point

Break point was based on the progressive-ratio schedule of reinforcement (Deroche-Gamonet et al. 2004). Drug availability was signaled by the blue cue light and the ratio of responses was increased after each infusion according to the following progression: 10, 20, 30, 45, 65, 85, 115, 145, 185, 225, 275, 325, 385, 445, 515, 585, 665, 745, 835, 925, 1025, 1125, 1235, 1345, 1465, 1585. The last completed ratio performed by the rat was referred to the breaking point. The test ended after either 6 h or elapsed 1h after the last cocaine infusion when the ratio is not completed. Animals (n = 19) were assigned to groups according to their performance in the last three CSA sessions (Fig. 17).

Cue-induced reinstatement

Following 3 days of the BP test, rats were subjected to extinction training (daily 2h session in the absence of both cue lights and cocaine infusions). Extinction was carried out until the extinction criterion was achieved (<20% of active nose pokes in the last three CSA sessions). Cue-induced reinstatement was tested on the next day after the final extinction session for 1h. The blue cue-light and the contingent white cue-light were presented under FR5 schedule. However, cocaine was not available. The number of nose pokes in both active and inactive holes was recorded.

Open field

Locomotor activity was assessed in a separate group of rats (n = 9). Distance traveled was tested in the open field (OF) apparatus made of dark PVC (51 × 51 × 50 cm). A camera above the apparatus recorded the animal's movements, which were analyzed using the BViewer software (Biobserve GmbH, Bonn, Germany). All animals were habituated to the experimental room 1 day prior to the test. Light was adjusted to ~15 lux (measured in the center of the square). Each animal was tested for 2h in the OF.

Sucrose preference

Sucrose preference was measured at the end of the study in all rats (n = 28) in a two-bottle choice paradigm. Baseline sucrose preference was monitored for a period of 48 h. To test the melatonin, animals were grouped according to their baseline preference, and bottles were weighted 1h and

24h after the availability of the bottles to the animals. Preference was calculated using the following equation: [sucrose consumed (g)/total liquid consumed (g)] × 100.

Data analysis

Data obtained from the BP, sucrose preference, and OF (total distance traveled) tests were analyzed using one-way analysis of variance (ANOVA). Two-way repeated measures ANOVA was applied for the analysis of distance traveled measured in 30-min intervals. Data from the cue-induced cocaine-seeking test was analyzed by a three-way ANOVA (factors were treatment group, nose pokes (active vs. inactive), and session (extinction vs. reinstatement)). Animals that did not extinguish or the number of inactive nose pokes exceeded active nose pokes by twofold or more were removed from the reinstatement test (n = 4). Whenever significant differences were found, post hoc Student Newman-Keuls test was performed. The chosen level of significance was $P < 0.05$. Data were analyzed using Statistica and GraphPad Prism softwares.

Results

Part I:

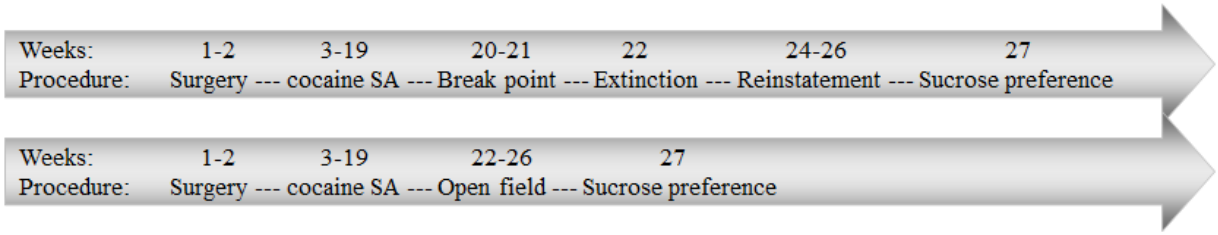


Figure 31 Time course of experiments.
Following catheter implantation, animals were submitted to 70 sessions of cocaine self-administration (CSA). Subsequently, rats were divided into two groups. The group A (n = 19) was tested in the break point and the cue-induced reinstatement tests, whereas the group B was tested in the locomotor activity (n = 9). Animals were reused to reach n = 8–10/ treatment condition using the Latin Square Design. Sucrose preference was measured in all animals at the end of the tests.

Melatonin reduced the motivation to self-administer cocaine

At the end of the 70 sessions of CSA, animals exhibited 432 ± 91 active nose pokes and 121 ± 38 inactive nose pokes (mean ± SEM of the last 3 CSA sessions). Rats were then subjected to the BP test. Analysis of BP data revealed that melatonin treatment reduced the motivation to self-administer cocaine. The break point was significantly lower in the melatonin-treated groups compared to the vehicle-treated group ($F_{(2,21)} = 3.98$, $P < 0.05$, Fig. 32-A). The number of active pokes was similarly reduced in animals treated with both doses of melatonin ($F_{(2,21)} = 4.08$, $P < 0.05$, Fig. 32-B). Post hoc analysis revealed significant difference between the higher dose of melatonin-treated group and vehicle-treated groups in both break point and number of active pokes. Treatment with lower melatonin dose significantly reduced the number of active nose-pokes, but there was no significant difference in the break point analysis. We have also performed a Pearson's correlation test between the number of nose pokes and the performance in the BP test. However, there was no correlation between these values ($P = 0.34$).

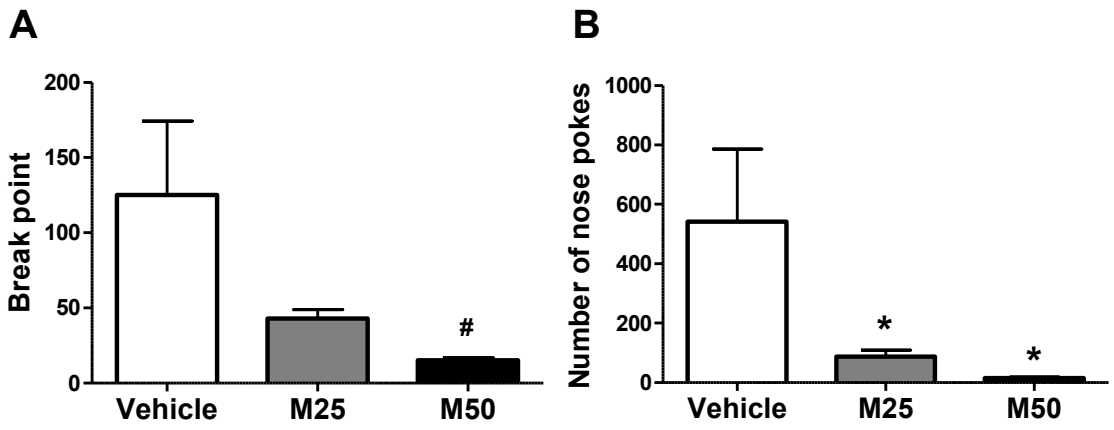


Figure 32 Melatonin effects on the motivation to take cocaine.
The last completed ratio (A) and the total number of nose pokes in the active hole (B) during the break point test. Vehicle, 25 mg/kg (M25) of melatonin or 50 mg/kg (M50) of melatonin was administered 30 min prior to the test (n = 8/ treatment condition). Data is presented as means ± S.E.M. #significant against all groups; *significant from vehicle-treated group, $P < 0.05$

Melatonin prevented relapse-like behavior

The number of operant responses progressively faded away across eight extinction sessions. Extinction criterion was achieved in the last three extinction sessions. Active nose pokes dropped to 62±10 nose pokes and inactive nose pokes dropped to 29±4, corresponding to 86% reduction in the active nose pokes compared to baseline CSA. Analysis of the cue-induced reinstatement data indicated a main effect of the treatment ($F_{(2, 54)} = 12.23$, $P < 0.001$), the sessions ($F_{(1, 54)} = 16.92$, $P < 0.001$), and the interaction treatment vs. sessions ($F_{(2, 54)} = 17.03$, $P < 0.001$). Post hoc test revealed significant differences between the sessions (extinction vs. reinstatement) in the vehicle-treated group, demonstrating that the cue presentation triggered cocaine seeking, measured as increased number of nose pokes in the active hole. Melatonin treatment, however, abolished this increase (Fig. 33-A). The number of active nose pokes in the melatonin-treated groups was not different from performance in the extinction. However, it was significantly lower compared to vehicle-treated rats. Number of nose pokes in the inactive hole was statistically lower in the animals treated with 50 mg/kg of melatonin compared to vehicle-treated animals (Fig. 33-B), indicating that this dose may produce side effects.

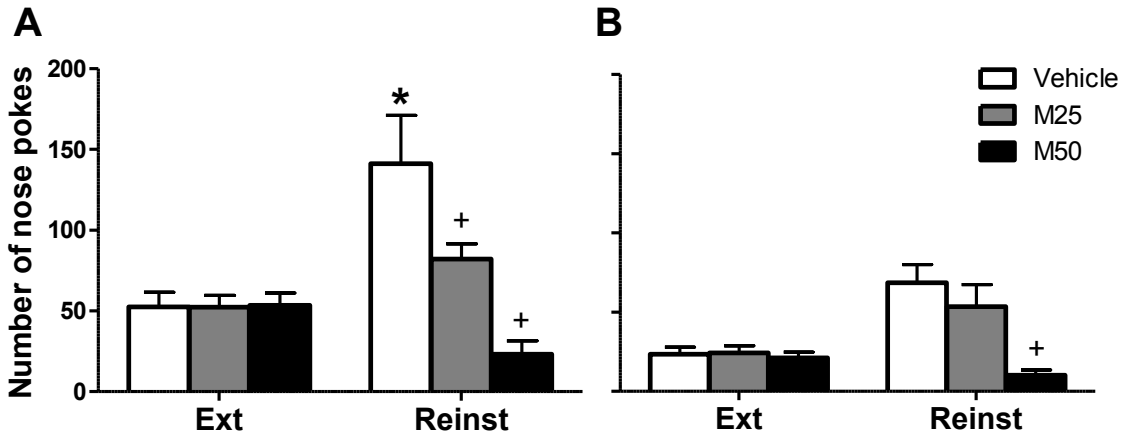


Figure 33 Melatonin effects in the cue-induced reinstatement test.
Number of active (A) and inactive (B) nose-pokes during the cue-induced reinstatement test. Vehicle, 25 mg/kg (M25) of melatonin or 50 mg/kg (M50) of melatonin was administered 30 min prior to the test (n = 10/ treatment condition). Data is shown as the number of nose pokes during the last three extinction sessions (Ext) and during the reinstatement test (Reinst). Graphs are presented as means ± S.E.M. *different from Ext; +different from vehicle-treated group in the reinstatement test, $P < 0.05$

Melatonin neither affected locomotor activity nor changed sucrose preference

Both doses of melatonin (25 and 50 mg/kg) tested in the present study did not produce significant changes on the animals' locomotor activity (Fig. 34). Analysis of the total distance traveled and the 30-min intervals in the OF test did not show significant differences between the treatment groups (P = 0.57).

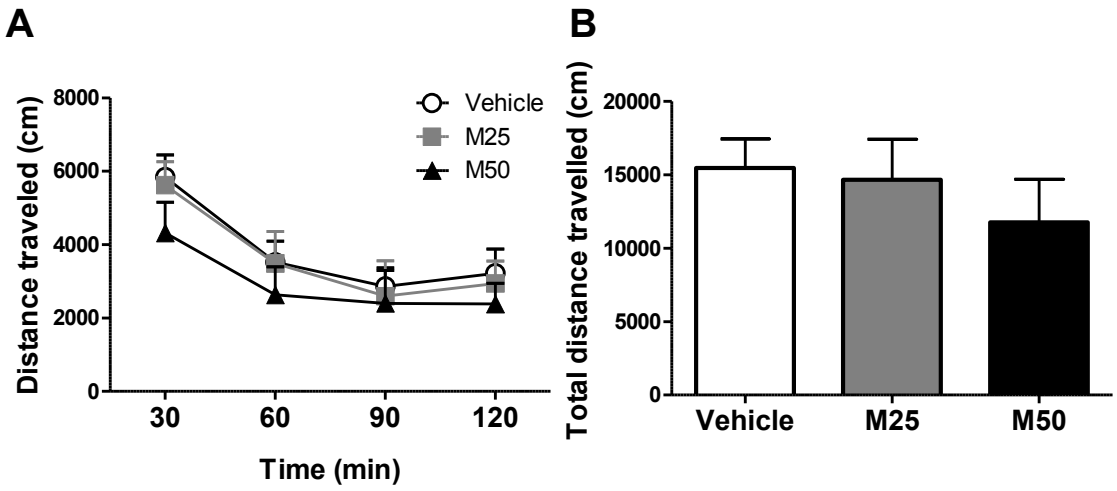


Figure 34 Melatonin effects on the locomotor activity.
Vehicle, 25 mg/kg (M25) of melatonin or 50 mg/kg (M50) of melatonin (n = 5–6/ treatment condition) was administered 30 min prior to the test. The data is shown as distance traveled in every 30 min interval (A) and the total distance traveled during the 2h testing (B). Data is presented as means ± S.E.M. No significant difference was found among treatment groups.

Similarly to the OF test, one-way ANOVA revealed no significant difference in sucrose preference between melatonin-treated and vehicle-treated groups. Neither 1h ($F_{(2,25)}$ = 0.03, P = 0.98, Fig. 35-A) nor 24h ($F_{(2,25)}$ = 1.49, P = 0.20, Fig. 35-B) after sucrose consumption were different, indicating that the doses of melatonin tested in the present study did not change the reinforcing properties of sucrose.

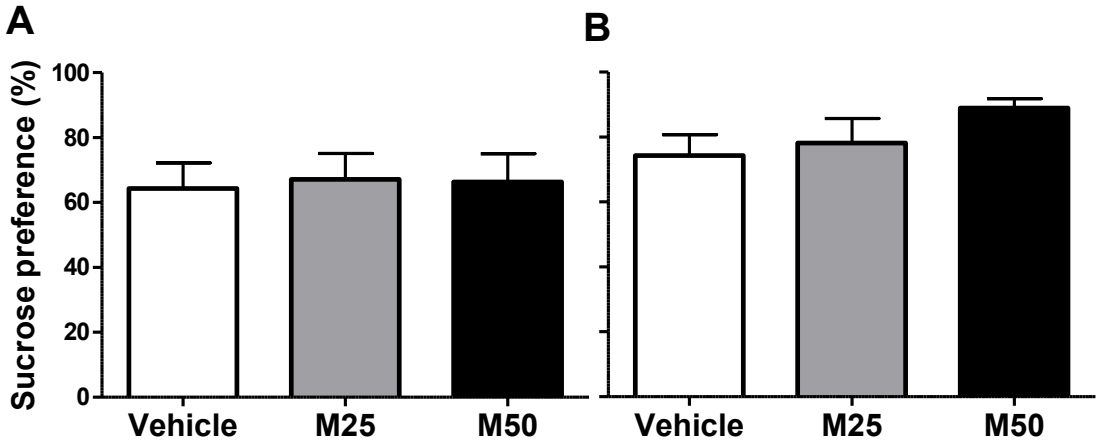


Figure 35 Melatonin effects on sucrose preference.
Sucrose preference was assessed in a two-bottle free-choice paradigm in chronic cocaine self-administered rats. Vehicle, 25 mg/kg (M25) of melatonin or 50 mg/kg (M50) of melatonin (n = 9–10 per treatment group) was administered 30 min prior to the test. Sucrose preference was measured 1h (A) and 24h (B) after availability of sucrose bottle. Data is presented as means ± S.E.M. No significant difference was found among treatment groups

Part II:

Melatonin effects on the cue-induced reinstatement test may be mediated by MT_1 subtype

To evaluate whether melatonin effects on cocaine-seeking behavior were provided by activation of MT_1 or MT_2 subtype, we used a combination of a selective MT_2 antagonist drug 4P-PDOT and melatonin in another batch of cocaine trained rats on cue-induced reinstatement test. Partial agonist of MT_2 (UCM-924) was also tested to confirm the results. UCM-924 was chosen because selective MT_1 antagonist drugs are unfortunately non-commercially available to date. Therefore, this is a preliminary study.

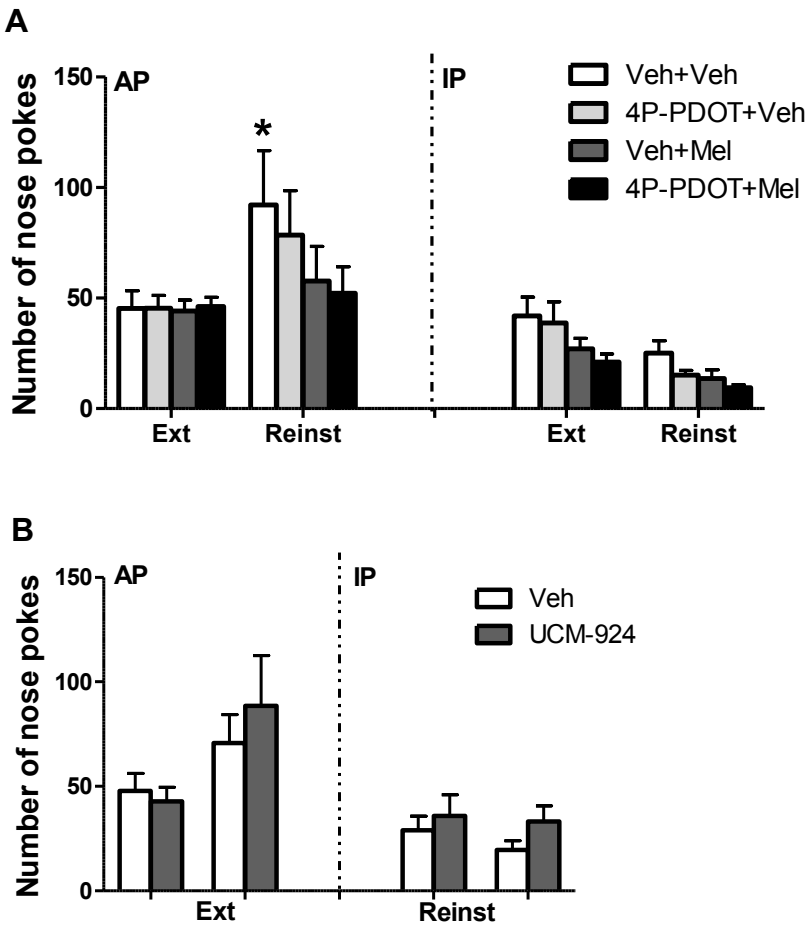


Figure 36. Effects of 10 mg/kg MT_2 antagonist (4P-PDOT) or 20 mg/kg MT_2 partial agonist (UCM-924) in the cue-induced reinstatement test

Number of active (AP) and inactive (IP) nose-pokes during the cue-induced reinstatement test. Vehicle (Veh) or 4P-PDOT was administered (s.c.) 15 min before melatonin (Mel, 40 mg/kg, i.p.) injection. Following 20 min from melatonin injection, animals were tested in the cue-induced reinstatement (Fig. A). Veh or UCM-924 (s.c.) was injected 20 min prior to the reinstatement test (Fig. B). Graphs are presented as means ± S.E.M. *different from Ext, $P < 0.05$

Discussion

The present study shows that melatonin administered within the first hours of the dark phase onset reduced both the cocaine seeking behavior and the motivation to take cocaine. The higher dose of melatonin (50 mg/kg) also decreased the number of inactive pokes during the cocaine

cued-reinstatement test, indicating that at this dose sedation may be seen. However, locomotor activity and sucrose preference were unaffected.

Dose-dependent effects of light and melatonin have previously been reported on alcohol and cocaine reinforcement. In short-term alcohol drinking rats, administration of low doses of melatonin produced an increase in voluntary alcohol drinking, whereas administration of melatonin antagonist GR128107 significantly reduced alcohol consumption (Crespi, 2012; Smith, 1980). Conversely, melatonin effects in cocaine reinforcement showed opposite direction to alcohol. Conditioned cocaine reinforcement in naïve rats was greater when the conditioning had been performed during the light phase, i.e., when melatonin levels are low (Akhisaroglu et al., 2004; Uz et al., 2003). Interestingly, in alcohol addicted rats, which were subjected to long-term (up to 1 year) alcohol exposure, agomelatine, a non-selective agonist of melatonergic receptors, as well as melatonin reduced the wanting and the relapse-like alcohol drinking (Vengeliene et al., 2015). Similar to Vengeliene studies, in our present study on cocaine addicted rats, which self-administered cocaine over three months, melatonin decreased motivation to self-administer cocaine and abolished the relapse-like behavior. We suggest that melatonin may produce distinct effects in naïve/ short treated-rats and in chronically treated-rats to drugs of abuse. The efficacy of melatonin in drug addicted animals could be explained by drug-induced reductions in MT_1/MT_2 receptors after long-term drug treatment regimens (Imbesi et al., 2006). Furthermore, melatonin effects in drug addicted rats seem to be selective for drugs of abuse-related behaviors as sucrose preference was not affected.

The underpinning mechanisms by which melatonin decreased the motivation and the drug-seeking responses need further investigations. A possible explanation, at least to some degree, is that melatonin could normalize the circadian activity, which is disrupted by long-term drug abuse (Emens and Burgess, 2015; Loganathan et al., 2018; Perreau-Lenz and Spanagel, 2008). Another possibility is the interactions between melatonin and dopaminergic and/or glutamatergic neurons in brain regions that play important role in drug-seeking behavior. Melatonergic receptors have been found in dopamine innervated structures (Khaldy et al., 2002; Luo and Aston-Jones, 2009; McClung, 2007; Uz et al., 2005b). Although, it is unclear how melatonin acts in the presence of

drugs of abuse or drug addiction, the administration of melatonin in a healthy state can reduce dopamine release in brain regions, such as striatum (Khaldy et al., 2002; Schiller et al., 2003; Zisapel, 2001). In addition, in vitro studies using rat striatum, melatonin also reduced glutamate dependent excitatory responses to stimulated neurons and suppressed nitric oxide synthase (NOS) activity (Castillo-Romero et al., 1993; Escames et al., 2001; Leon et al., 1998), attenuating an increase in extracellular glutamate. Specifically, glutamatergic and dopaminergic neurons in the ventral and dorsal striatum can modulate drug-seeking behavior induced by conditioned cues (Tzschentke and Schmidt, 2003) and reinstatement of cocaine-seeking (Hulka et al., 2016; Kalivas, 2009). The mechanisms by which melatonin exerts effects in drug addiction state is yet to be clarified. Nevertheless, it is likely that melatonin may be modulating dopaminergic and glutamatergic signaling to produce the observed effects in this study.

GABAergic system has also been demonstrated to be target by melatonin. Earlier studies have shown that almost every neuron in the suprachiasmatic nucleus contains GABA (Golombek et al., 1996; Okamura et al., 1989). The downstream effect depends on the melatonin receptor subtype and brain region to be activated. For instance, Wan et al. (1999) found increased $GABA_A$ receptor-mediated currents via the MT_1 subtype in the rat suprachiasmatic nucleus, but a reduction of the current amplitude via MT_2 in CA1 neurons of the hippocampus. The low number of inactive nose-poke during the reinstatement test, an indication of sedative effects, may have caused by the activation of GABAergic neurons by melatonin. Nevertheless, whether melatonin and GABAergic neurons are playing a role in drug addiction has yet to be clarified.

In the Part II of the study, we assessed whether melatonin effects are mediated by MT_1 or MT_2 receptor. Co-administration of MT_2 antagonist 4P-PDOT and melatonin (Fig. 22-A) on cue-induced reinstatement test as well as results from administration of MT_2 receptor antagonist UCM924 (Fig. 22-B) together suggest that the abolishment produced by melatonin administration on cocaine seeking is mediated by MT_1 activity. However, the interpretation of the results is limited because: (1) partial agonist of MT_2 receptor UCM924 was used instead of MT_1 receptor antagonists because this last is not commercially available to date. This certainly limits the interpretation of the present data on pharmacological effects of melatonin; (2) MT_2 antagonist 4P-

PDOT show very low solubility in water and other polar solvents. Therefore, 80% dimethyl sulfoxide (DMSO) was used as recommended by Dr. Comai. However, a DMSO concentration ranging between 1 and 20% is commonly used in pharmacological tests due to unclear toxic effects of DMSO as well as no guidelines are available restricting its concentration. However, recent studies have shown that DMSO concentrations over 10% produced pore formation in plasma membrane (Galvao et al., 2014) and, *in vitro* rat hippocampal culture preparation, DMSO produced neuronal loss at concentrations as little as 0.5% and 1% (Hanslick et al., 2009). The high concentration of DMSO (70 – 80%) used in our study appeared to also disturb cocaine cue-induced reinstatement (Fig. 36 A, B), as the vehicle-treated rats failed to reinstate.

In conclusion, administration of melatonin significantly reduced the cocaine cue-induced reinstatement in chronic cocaine experience rats. Although selective MT_1 and MT_2 drugs with good solubility in water are yet to be developed in order to confirm the present results, our preliminary data indicates that melatonin effects on cocaine seeking behavior is mediated by MT_1 subtype. The present data suggests that melatonin could be helpful to prolong cocaine withdrawal period and to assist cocaine addiction treatments.

5.3 GluN3A-containing NMDA receptors

NMDA receptors (NMDARs) are glutamate-gated ion channels, which have central roles in learning, synaptic plasticity and development. NMDARs have heterotetrameric assemblies and therefore constituted of four subunits, including GluN1 subunit that is mandatory, in combination with GluN2 (GluN2A, GluN2B, GluN2C and GluN2D) and/or GluN3 (GluN3A and GluN3B) subunits. In the extracellular region of GluN1 and GluN3 subunits there is a glycine or d-serine binding domain and in GluN2 subunit, a glutamate-binding domain (Perez-Otano et al., 2016). Different subunit compositions determine the receptor kinetics and thus the intracellular signaling cascades of the NMDAR (Yuan et al., 2013), which produce distinct biophysical, pharmacological and signaling attributes. Classical NMDARs contain two GluN1 and two GluN2 subunits, and are highly permeable to Ca^{2+}, generating 'high-conductance' during the channel openings, with high sensitivity to Mg^{2+} blockade. Incorporation of GluN3 subunit, such of

triheteromeric GluN1/GluN2/GluN3 receptors, produce a dramatic decrease in Mg^{2+} blockade as well as in Ca^{2+} permeability, producing a smaller single-channel conductance, a lower open probability (Rauner and Kohr, 2011), and longer mean opening time compared with classical NMDARs (Henson et al., 2010; Pachernegg et al., 2012). Insensitivity to Mg^{2+} may explain why GluN3-containing NMDARs can be active while the membrane is depolarized (Burzomato et al., 2010; Pina-Crespo et al., 2010).

Earlier studies have demonstrated that GluN3A-containing NMDARs destabilize synapses (Perez-Otano et al., 2016). Studies on GluN3B subunits are limited and little information are known for GluN3B-containing NMDARs. Recently many neuropsychiatric disorders have been linked to synaptic defects (synaptopathies) and NMDAR dysfunction, expressed either as altered subunit expression, trafficking, localization or activity (Endele et al., 2010; Lau and Zukin, 2007; Mony et al., 2009; Traynelis et al., 2010). Alteration in NMDAR subunits composition have been found in psychiatric conditions, including stroke, neurodegenerative diseases, schizophrenia, and chronic pain (Henson et al., 2010; Paoletti et al., 2013). This is due to the plasticity of NMDARs subunit composition, which changes in response to neuronal activity or sensory experiences. These changes can occur within minutes and have large influences on the functioning of synapses and networks. Few studies have investigated the link between NMDARs subunit composition and drugs of abuse. Yuan et al. (2013) have demonstrated that acute cocaine injections produce insertion of NMDARs-containing GluN3A and GluN2B subunits. Furthermore, these GluN3A-containing NMDARs appear to be necessary for the expression of cocaine-evoked plasticity of AMPARs (Yuan et al., 2013). However, whether GluN3A-containing NMDARs play a role in drug addiction is yet to be clarified. The aim of this study was to assess whether decrease of GluN3A-containing NMDARs in the anterior cingulate cortex would attenuate the addiction-like behaviors in cocaine-addicted rats. The region was chosen based on the previous studies from Prof. Spanagel's group (unpublished data), where mRNA expression of GRIN3A, the gene encoding GluN3A, was found increased in ACC of human post-mortem brain samples of alcoholics.

Material and methods

Animals and cocaine self-administration

Animals were trained in the 0/3crit model of cocaine addiction. For this experiment, 1crit and 2crit were used.

Drugs

Cocaine hydrochloride (Sigma Aldrich, Taufkirchen, Germany) was dissolved in sterile saline.

Stereotaxic injection

Following about 55 CSA training sessions, animals were subjected to stereotaxic surgery for virus injection. The GluN3A knocking down virus (AAV9-CMVEGFP-shGluN3A virus) or control virus (AAV9-GFP-scrmb-shRNA) was injected in the ACC (AP: +3.0; ML: ±0.5; DV: -2.3). Animals were deeply anesthetized with isoflurane (~400 ml/L, induction: 5%, maintenance: 2- 2.5%) and placed in a stereotaxic head-holder (David Kopf Instruments, Tujanga, CA, USA). Craniotomies were made directly above the target region of the brain and injected a volume of 2 ul / hemisphere. Both knockdown and control virus were kindly provided by Dr. Isabel Perez-Otano, University of Navarra, Spain.

Addiction-like behaviors

Following 3-4 weeks from stereotaxic surgery, animals were retrained in the CSA and the three addiction-like behaviors tested, as follow:

Motivation for cocaine was assessed in a break point (BP) test. Blue and white cue lights were ON during the test and the progressive ratio of reinforcement was schedule as follow: 10, 20, 30, 45, 65, 85, 115, 145, 185, 225, 275, 325, 385, 445, 515, 585, 665, 745, 835, 925, 1025, 1125, 1235, 1345, 1465, 1585. The test ceased either after 6h or when the ratio was not completed within 1h period. The last completed ratio performed by the rat was used as representative of their motivation for taking cocaine.

Persistence of drug-seeking was analyzed as the mean active nose-pokes during NO-drug periods in the last three CSA training sessions prior to the BP test.

Resistance to punishment was assessed by pairing cocaine infusion and foot shocks (0.2 mA, 1s). In addition to the blue and white cue lights, a green cue-light was turned on at FR1 to indicate the

presence of a shock. Two foot-shocks were delivered, one foot shock at completion of the 4th nose-poke, and another shock delivered at the 5th nose-poke, which was paired with cocaine infusion. The test lasted 40 min and the criterion was expressed as percentage of cocaine infusions earned in relation to the baseline training.

Data analysis

Performance in the addiction-like behaviors was assessed using t-test for comparison between control virus-injected and knockdown virus-injected rats. Performance in the CSA over the training sessions was analyzed using repeated-measures analysis of variance (ANOVA). Whenever significant differences were found, Student Newman-Keuls post-hoc test was performed. The chosen level of significance was $P < 0.05$.

Results

Knocking down of GluN3A-NMDAR in the Anterior Cingulate Cortex did not change the addiction-behaviors

GluN3A-knowckdown virus and control-virus were injected in the anterior cingulate cortex following ~55 CSA training sessions (n=7-8/group). Following three to four weeks from stereotaxic surgery, animals were retrained in the CSA (Fig. 37-A-C) and addiction-like behavior tested (Fig 37 D-F). Motivation to seek the drug was assessed on the retrained CSA session 5 of after the virus expression, in the break point test (D). Persistence of cocaine-seeking expressed by the sum of active nose-pokes during the no-drug periods, analyzes between retrained CSA sessions 2 and 4 during retraining (E). Resistance to punishment expressed as percent cocaine infusions paired with a foot-shock in relation to baseline infusion, assessed during the retrained CSA session 10 (F). No significant difference was found in any of the assessments ($P > 0.05$).

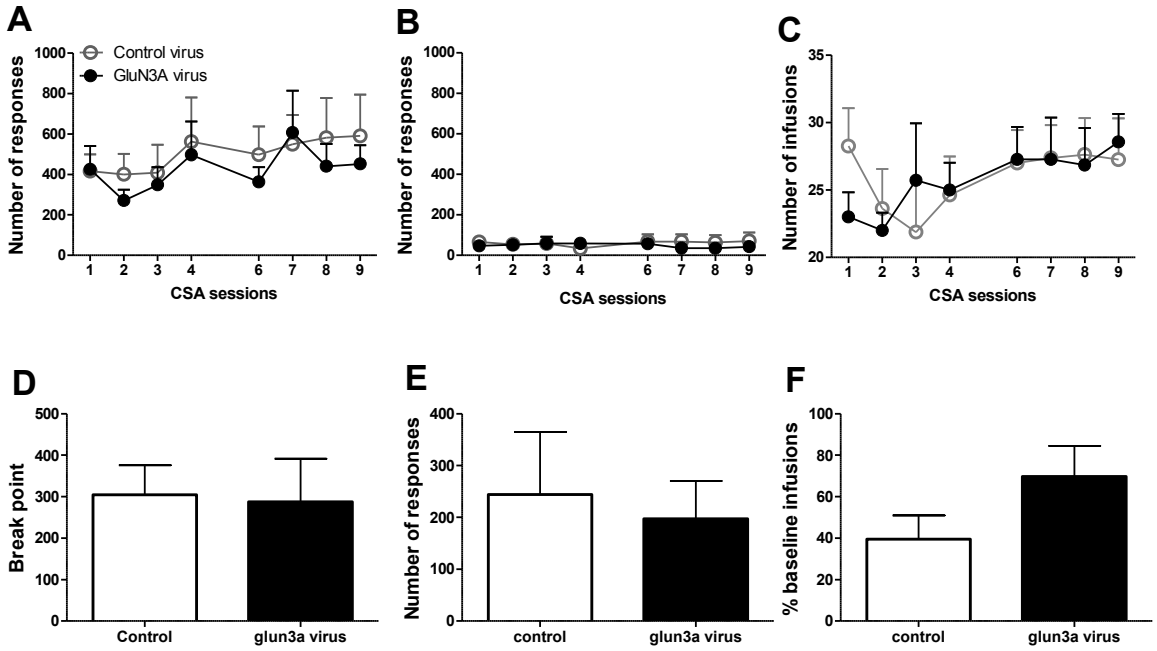


Figure 37 Effects of GluN3A-NMDAR knockdown in cocaine self-administration and addiction-like behaviors.

Number of active (A), inactive (B) and cocaine infusions (C) during the retraining of cocaine self-administration following 3-4 weeks from virus injection. (D) Motivation to seek cocaine, (E) persistence of cocaine-seeking, and (F) resistance to punishment. Data represent mean ± SEM; no significant difference was found between groups ($P>0.05$).

GFP expression of the GluN3A-knockdown and control viruses in the Anterior Cingulate Cortex

Virus expression was checked in the ACC following the behavioral assessments. Animals were anesthetized and perfused with 4% PFA. Brains were collected, frozen and sliced in cryostat for ~40 um thickness. Pictured were collected with fluorescence microscope (Zeiss, Göttingen, Germany). Expression of both control and GluN3A viruses were strong in the ACC, as it is shown in the Fig. 38.

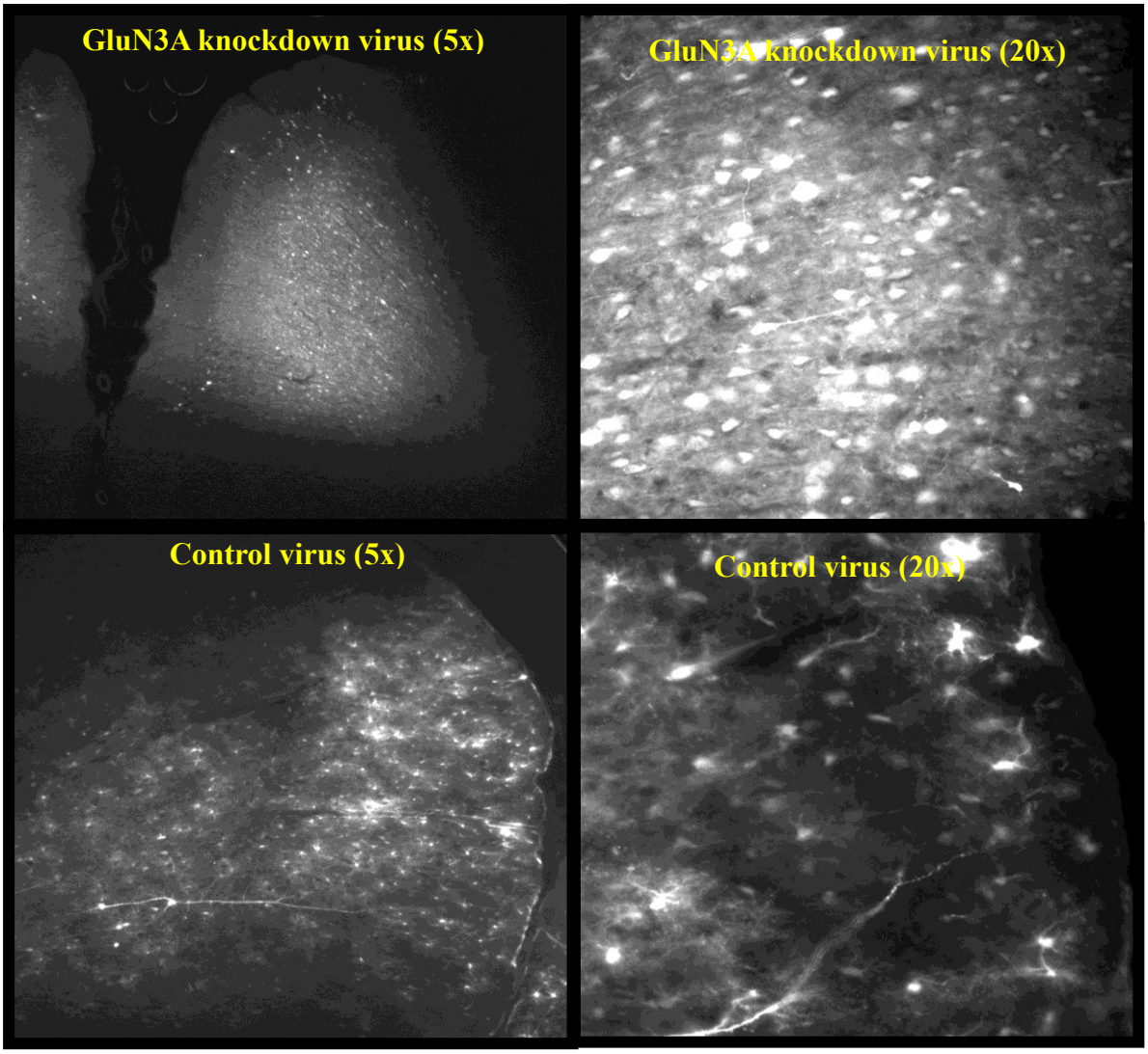


Figure 38. GFP expression of GluN3A knockdown virus and control virus in the anterior cingulate cortex of cocaine experience rats.
Pictures were collected in fluorescence microscope at 5x and 20x magnification.

Discussion

The present study demonstrates that knocked down of GluN3A-containing NMDARs in the Anterior Cingulate Cortex neither affected the performance in cocaine self-administration nor the addiction-like behaviors.

In a study from Spanagel's group (unpublished data), increased mRNA expression of GRIN3A, the gene encoding GluN3A, was found in ACC of human post-mortem brain samples of

alcoholics. ACC displays functionally distinct cells that participate in detecting targets (Frith et al., 1991), influence motor responses (Badgaiyan and Posner, 1998; Paus et al., 1993; Turken and Swick, 1999), encode reward values (Mesulam, 1990), signal errors (Gehring and Knight, 2000; Luu et al., 2000), and participate in reward-based decision making (Bush et al., 2002). Brain imaging studies have revealed ACC activation during drug (cocaine, metamphetamine or alcohol) intoxication, bingeing, and craving, suggesting that this brain region may drive the drug seeking behavior (Goldstein and Volkow, 2002; Kosten et al., 2006). Although the ACC have been implied in limbic-regulated responses to reward and pleasure (Goldstein and Volkow, 2002), the increased mRNA expression of GRIN3A in the ACC of alcoholics (as mentioned above) may not be the in the control of addictive behaviors. Animals that received either control-virus or knockdown-virus in this brain region did not significantly differ in their performance during cocaine self-administration and in any of the DSM-based addiction-like behavior tests. A possible explanation for this result is either the increased GluN3A-containing NMDARs is not driving the addiction-like behaviors in cocaine abusers or dysfunction of other brain regions may play bigger role in the control of addiction behaviors.

Several corticolimbic structures are activated during cocaine craving and seeking, such as prelimbic cortex, orbitofrontal cortex, ventral and dorsal striatum, as well as amygdala (Backstrom and Hyytia, 2007; Cannella et al., 2013; Di Ciano and Everitt, 2004b; Ito et al., 2002; Kalivas and McFarland, 2003; Shaham et al., 2003). It is likely that recruitment of most, if not all, of those brain regions are necessary to affect the addiction behaviors to cocaine. Conversely, recent evidences have suggested that GluN3A-containing NMDARs may hold a protective role in acute conditions. Wang et al. (2013) have demonstrated that overexpression of GluN3A, but not GluN3B, in brain ischemia and hypoxia produced a neuroprotective effects during the ischemia and hypoxia processes. They also demonstrated that the neuroprotective mechanisms of GluN3A involves blockade of augmentation of intracellular Ca^{2+} concentration, suppression of hydroxyl radicals and NO generation. Lee et al. (2015) have also demonstrated that increased GluN3A-containing NMDARs produced neuroprotective effects in cerebral ischemia against excitotoxic insults in cortical neurons in the adult brain. Although acute and chronic conditions may differ, the plastic ability of the NMDA subunits composition suggests that overexpression of GluN3A in

the cerebral cortex may likely be protective rather than damaging mechanism in chronic conditions, such as addiction. The knowledge that NMDAR subunit assembly changes over time after stress, from minutes to hours, also implies that it may normalize following stabilization. Further studies are needed to clarify this hypothesis and the role of GluN3A-containing NMDARs in addiction.

In conclusion, the knockdown of GluN3A-containing NDMARs in the ACC appears to not play a role in cocaine addiction-like behaviors. Other brain regions as well as the plastic NMDAR subunits assembly need further investigation to clarify the role of GluN3A-containing NMDARs overexpression in drug addiction.

Chapter 6: General Discussion

Although numerous studies have documented functional and structural changes on brains of drug abusers, the neurobiological changes that produce the compulsive drug-taking and –seeking are unclear. To better understand the individual differences and the brain changes caused by chronic drug use from those related to addiction, investigating brain differences between drug users that develop addiction, ~18% for psychostimulant abusers (Anthony et al., 1994; Nutt et al., 2007), from those resilient to addiction is a better research approach than typical studies comparing healthy controls and drug abusers. The 0/3crit animal model of cocaine addiction identify rats displaying different levels of addiction-like behaviors, despite their similarities in age, genetic background, environmental conditions, and, most importantly, cocaine infusions. Strikingly, similar to epidemiological studies, about 18% of rats trained in this model develop addiction-like behaviors. Hence, the aim of the present thesis was to investigate pre-existing and acquired brains changes in cocaine addicted-like (3crit) and non-addicted-like (0crit) rats throughout the course of drug use. Salience of conditioned stimuli was assessed in the Pavlovian to instrumental transfer paradigm as an attempt to measure vulnerability or predictability for addiction. In addition, novel drugs to attenuate cocaine-seeking behavior were also assessed in this thesis.

Preliminary results from the longitudinal MRI study (chapter 2) revealed increased grey matter (GM) volume in several brain regions in 3crit rats. The affected regions were mainly in those related to rewarding effects of cocaine, such as Prl, Cg, NAc, CPu, VP, GP, and SN. Conversely, 0crit and control groups showed similar brain changes through time, except for an increase in CPu GM volume in 0crit rats. It appears that 0crit rats are able to maintain normal brain development through age despite the chronic cocaine use, which may indicate counteracting mechanisms to protect from toxic effects of cocaine or, alternatively, 3crit rats may be more sensitive to cocaine toxicity. DTI and FDG-PET results seem to support the first hypothesis. 0crit rats showed higher anisotropic movement of water in the Zona Incerta (ZI), a brain region that regulates brain structures that modulate adaptive behavior to unexpected sensory stimuli, such as dorsal striatum and dorsomedial thalamus. The increased fractional anisotropy in ZI may reflect an efficient communication with the connecting brain regions, possibly leading to more flexible

behavioral responses in order to adapt the behavior to sensory stimuli, such as cocaine-associated stimuli. Functional assessment with FDG-PET showed higher baseline glucose utilization in the CPu and mPFC in 0crit compared to control rats. The dorsal striatum (DS), which is part of caudate and putamen, is a brain region essential for habitual behavior (Everitt and Robbins, 2016). Therefore, the increased GM volume of CPu in both 0crit and 3crit rats suggest that habit is not unique for addiction. The extensive CSA training may have caused it as increased GM volume have been reported in individuals who practice regular physical activity (Erickson et al., 2010). The enhanced CPu activity in 0crit could have been produced by higher microstructural integrity in ZI. As above mentioned, ZI communicates with dorsal striatum and the enhanced fractional anisotropy in ZI could be enhancing CPu activity. Regarding the increased baseline activity of mPFC in 0crit rats, it suggests that this brain region may also contribute to enhanced behavioral control.

The mPFC were differently activated in 0crit and 3crit groups during cue-induced reinstatement (chapter 3). The IEG Arc was expressed substantially lower in the mPFC, specifically in the IFL, in the 3crit compared to 0crit rats. IFL is suggested to guide the "stop" behavior, while Prl cortex serves as a "go" behavior (Gourley and Taylor, 2016). Although this is purely speculative due to the lack of difference in the Arc expression between Prl and IFL regions, they appear to go in opposing directions in the 3cri group. This could indicate that during cocaine seeking behavior 3crit rats not only show difficulty to stop cocaine seeking response due to impaired IFL function, but also be strongly influenced by the Prl region. Both Prl and IFL are part of the mPFC and dysfunction of this brain region has been previously reported in psychostimulant abusers (Goldstein and Volkow, 2011), supporting the present findings. Nevertheless, it would be interesting to clarify whether the neuronal activation in this brain region decreased in 3crit rats or increased in 0crit rats.

Although it is well established that conditioned stimuli play an important role in eliciting drug craving and seeking behaviors, 0crit and 3crit rats showed similar Pavlovian to instrumental transfer as well as similar acquisition of instrumental learning (chapter 4). These results suggest that learning capacity and salience to conditioned stimuli neither are vulnerability hallmarks nor

it can predict severity of substance use disorder as proposed by Garbusow et al. (2016). Nevertheless, there was a robust positive correlation between performance in the PIT and in the CSA, indicating that it can rather predict cocaine taking behavior.

Finally, pharmacological assessments on cocaine-seeking behavior were also studied (chapter 5). Among the drugs tested in this thesis, positive allosteric modulator of $GABA_B$ receptors abolished cue-induced reinstatement and showed reduced unwanted side effects compared to $GABA_B$ receptor agonists, indicating to be a better alternative than agonists. In addition, the effects of melatonin were surprisingly promising because it not only abolished the cocaine-seeking behavior, but also substantially reduced the motivation to take cocaine without affecting natural reward consumption (sucrose) and locomotor activity. It also demonstrates that circadian rhythm is actively involved in drug addiction and melatonin may be a helpful medication for treatments of cocaine abusers.

In summary, the present thesis shows that 0/3crit animal model is a great translational model for drug addiction research as the changes produced by addiction in addicted-like rats correlate with clinical studies. Further, 0crit rats may also be an interesting group to investigate, as they maintained normal brain development despite the chronic cocaine use. The higher microstructural integrity in the brain region that regulates adaptive behavior and the increased mPFC activity could be contributing to controlled drug-seeking and-taking. Despite the lack of differences in the behavioral expression between 0crit and 3crit in the Pavlovian to instrumental conditioning, learning ability and salience to conditioned stimuli cannot predict substance use severity, indicating that brain structural and functional changes are rather relevant factors leading to addiction behaviors. Finally, positive allosteric modulator of $GABA_B$ receptors and regulation of the circadian rhythm by administration of melatonin appears to be promising targets to assist treatments of psychostimulant abusers.

Outlook

The outlook for this thesis is as follow:

- Analysis of fMRI longitudinal data;
- Quantification of Arc expression in rats subjected to different length of cocaine self-administration treatments, e.g. short-term and sub chronic treatments. This could clarify how the neuronal activity changes over the course of drug use;
- Electrophysiology analysis of the IFL regions in both 0crit and 3crit rats could provide additional information about the synaptic plasticity in this brain area;
- Complete the 0crit and 3crit group size for the dendritic spines analysis;
- Further investigate the pharmacological mechanisms of melatonin effects on abolishment of cocaine seeking behavior with MT_1 antagonists, which is not commercially available to date.

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