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Voluntary cocaine or sugar intake induce neuroadaptations of the endocannabinoid system in reward-related brain regions

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Abstract

Occidental countries currently face an epidemic of obesity and related diseases. As eating disorders and drug addiction are both complex pathologies inducing long-term neuroadaptations, we investigated common alterations induced by either sugar or cocaine intake in reward-related brain regions. We focused our research on the endocannabinoid and opioid systems, as both systems are expressed in the central nervous system and play a crucial role in drug reward and food intake.

We first analyzed transcriptional regulations of components of the endocannabinoid and opioid systems in the prefrontal cortex, nucleus accumbens, dorsal striatum, hippocampus and ventral tegmental area following cocaine self-administration in rats. We found marked mRNA increase of several components of both systems in the hippocampus along with enhanced functional activity of both cannabinoid receptor 1 and mu opioid receptor. Interestingly, these changes were associated with an enhancement of the endocannabinoid levels in the hippocampus. We also investigated whether transcriptional regulation of the endocannabinoid and opioid systems upon cocaine could be under the control of epigenetic processes. We found activating histone modifications at endocannabinoid enzymes, but not at CB1 or mu receptors in the hippocampus. Interestingly, cocaine self-administration altered chromatin interactions at CB1 promoter locus, suggesting the involvement of other epigenetic mechanisms such as DNA methylation. Altogether, our findings suggest that voluntary cocaine intake strongly altered the endocannabinoid and opioid systems in the hippocampus, a structure highly involved in learning and context-associated reward memory.

Using a model of sucrose binge-like intake in rats, we investigated the neuroadaptations of the endocannabinoid system. Globally, this model induced fewer modifications compared with cocaine, both at the transcriptional level and the endocannabinoid tone. However, we observed a similar CB1 gene expression increase in the nucleus accumbens, highlighting a role for accumbal CB1 in reward and potential commonalities between binge-eating and cocaine addiction. To broaden our transcriptional study, we conducted an RNA-Seq analysis in this brain structure and identified gene expression regulations associated to both drugs of abuse and eating disorders.

Overall, our results highlight the hippocampus as a highly involved brain site following cocaine use. Moreover, our work sheds light on epigenetic mechanisms regulating the endocannabinoid system. More importantly, we demonstrate that a binge-like intake of sucrose induced similar transcriptional adaptations to that of voluntary cocaine intake in the nucleus accumbens. These findings may pave the way to new therapeutic targets for addictive behaviors. In this context, I also participated to two other projects examining (i) the potential of an orthosteric glutamatergic agonist as a therapeutic tool for reducing motivation to consume cocaine and (ii) the impact of the activation of another cannabinoid receptor, CB2, in epigenetic processes.

Acknowledgments

Tout d'abord, je remercie le professeur Jean-Christophe Cassel pour m'avoir accueilli au LNCA. Ces 4 années de travail resteront à jamais très spéciales pour moi, en particulier les parties de pétanque endiablées.

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"Time can but make it easier to be wise though now it seems impossible, and so all that you need is patience", William Butler Yeats

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Abbreviations

2-AG : 2-arachidonoylglycerol

5-HT : serotonin

AEA : anandamide

AgRP : agouti-related peptide

AM251 : 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3 carboxamide

AM281 : 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide

AM630 : 6-Iodopravadoline

AMT : anandamide membrane transporter

Amy : amygdala

AN : anorexia nervosa

Arc : arcuate nucleus

BED : binge eating disorder

BN : bulimia nervosa

BChE : butyrylcholinesterase

BLA : basolateral nucleus of the amygdala

CART : cocaine-amphetamine-regulated transcript

Cas9 : CRISPR-associated protein 9

CB1 : cannabinoid receptor 1

CB2 : cannabinoid receptor 2

CB2xP : CB2 overexpression

CBD : cannabidiol

CeA : central nucleus of the amygdala

CNS : central nervous system

Cocaine-SA : cocaine self-administration

COMT : catechol-O-methyltransferase

CP55,940 : 2-[(1R,2R,5R)-5-Hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol

CpG : cytosine-phosphate-guanine

CPP : conditioned place preference

CREB : cAMP response element-binding

CRISPR : clustered regularly interspaced short palindromic repeats

CYP3A4 : cytochrome P450 3A4

DAGLα : diacylglycerol lipase alpha

DAT : dopamine transporter

DNAm : DNA methylation

DNMT : DNA methyl transferases

DSI : depolarization-induced suppression of inhibition

DSE : depolarization-induced suppression of excitation

DSM5 : diagnostic and statistical manual of mental disorders (fifth edition)

eCB : endocannabinoid

eCB-STD : eCB-mediated short-term depression

eCB-LTD : eCB-induced long-term depression

ECS : endocannabinoid system

EGR : early growth response

EMT : endocannabinoid membrane transporter

ERK : extracellular signal-regulated kinase

EtOH : ethanol

FAAH : fatty acid amide hydrolase

GPCR : G protein-coupled receptor

GR : Glucocorticoid receptor

hCE-1 : liver carboxylesterase 1

HDAC2 : histone deacetylase 2

HFD : high fat diet

HPC : hippocampus

HU-308 : [(1R,2R,5R)-2-[2,6-Dimethoxy-4-(2-methyloctan-2-yl)phenyl]-7,7-dimethyl-4-bicyclo[3.1.1]hept-3-enyl]methanol

HYP : hypothalamus

JZL184 : 4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate

JWH133 : (6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro -6,6,9-trimethyl-6H dibenzo[b,d]pyran

LDX : lisdexamfetamine

LHb : lateral habenula

LTP : long term potentiation

MAGL : monoacylglycerol lipase

MAO : monoamine oxidase

MeCP2 : the methylated DNA-binding protein 2

miRNAs : microRNAs

MOP : Mu opioid receptor

NAc : nucleus accumbens

NAPE-PLD : N-arachidonoyl phosphatidylethanolamine – phospholipase D

ncRNAs : non-coding RNAs

NET : norepinephrine transporter

NFκB : nuclear factor kappa-light-chain-enhancer of activated B cells

NPY : neuropeptide Y

OS : opioid system

PFC : prefrontal cortex

POMC : pro-opiomelanocortin

PR : progressive ratio

RDoC : Research domains criteria

RMTg : rostromedial tegmental nucleus

RNA-Seq : RNA sequencing

SCN : suprachiasmatic nucleus

SERT : serotonin transporter

SGIP1 : Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1

SNPs : single-nucleotide polymorphisms

SR144528 : 5-(4-Chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-1H-pyrazole-3-carboxamide

TH : tyrosine hydroxylase

THC : Δ9-tetrahydrocannabinol

TRPV1 : transient receptor potential cation channel subfamily V member 1 also known as capsaicin receptor and vanilloid receptor 1

URB597 : cyclohexylcarbamic acid 3-carbamoyl biphenyl-3-yl ester

VTA : ventral tegmental area

VMAT2 : monoamine vesicle transporters

WIN55,212 : (11*R*)-2-Methyl-11-[(morpholin-4-yl)methyl]-3-(naphthalene-1-carbonyl)-9-oxa-1-azatricyclo[6.3.1.$0^{4,12}$]dodeca-2,4(12),5,7-tetraene

Introduction

A. Introduction

I. Addiction to drugs of abuse: an overview

1. Definition and diagnostic

Addiction is a neuropsychiatric disease characterized by compulsive and uncontrolled use of a psychoactive substance, harmful use, tolerance and withdrawal. Drugs of abuse such as alcohol, tobacco, psychostimulants, cannabinoids and opioids are induce substance use disorder (American Psychiatric Association, 2013). According to the diagnostic and statistical manual of mental disorders (fifth edition) (DSM5), individuals with substance use disorder are diagnosticated using 11 criteria related to behavioral and neurobiological changes:

- Using more of a substance than planned, or using a substance for a longer interval than desired
- Inability to cut down despite desire to do so
- Spending substantial amount of the day obtaining, using, or recovering from substance use
- Cravings or intense urges to use
- Repeated usage causes, or contributes to, an inability to meet important social, or professional obligations
- Persistent usage despite user's knowledge that it is causing frequent problems at work, school, or home
- Giving up or cutting back on important social, professional, or leisure activities because of use
- Using in physically hazardous situations, or usage causing physical or mental harm
- Persistent use despite the user's awareness that the substance is causing or at least worsening a physical or mental problem
- Tolerance: need to use increasing amounts of a substance to obtain its desired effects
- Withdrawal: characteristic group of physical effects or symptoms that emerge as amount of substance in the body decreases

One of the novelties brought by the DSM5 in the description of addiction is the severity of the substance use disorder in individuals over a 12 months period:

- Mild: 2-3 symptoms
- Moderate: 4-5 symptoms
- Severe: 6 ≥ symptoms

The National Institute of Mental Health introduced another tool to help researchers entitled "research domains criteria (RDoC)" (Insel *et al.*, 2010). RDoC enable researchers to override the flaws in DSM5, thus, both can be used concurrently (Lamontagne & Olmstead, 2019). More recently, the world health organization released the 11th version of the international classification of diseases relying on similar criterions to those from DSM5 (World Health Organization, 2018).

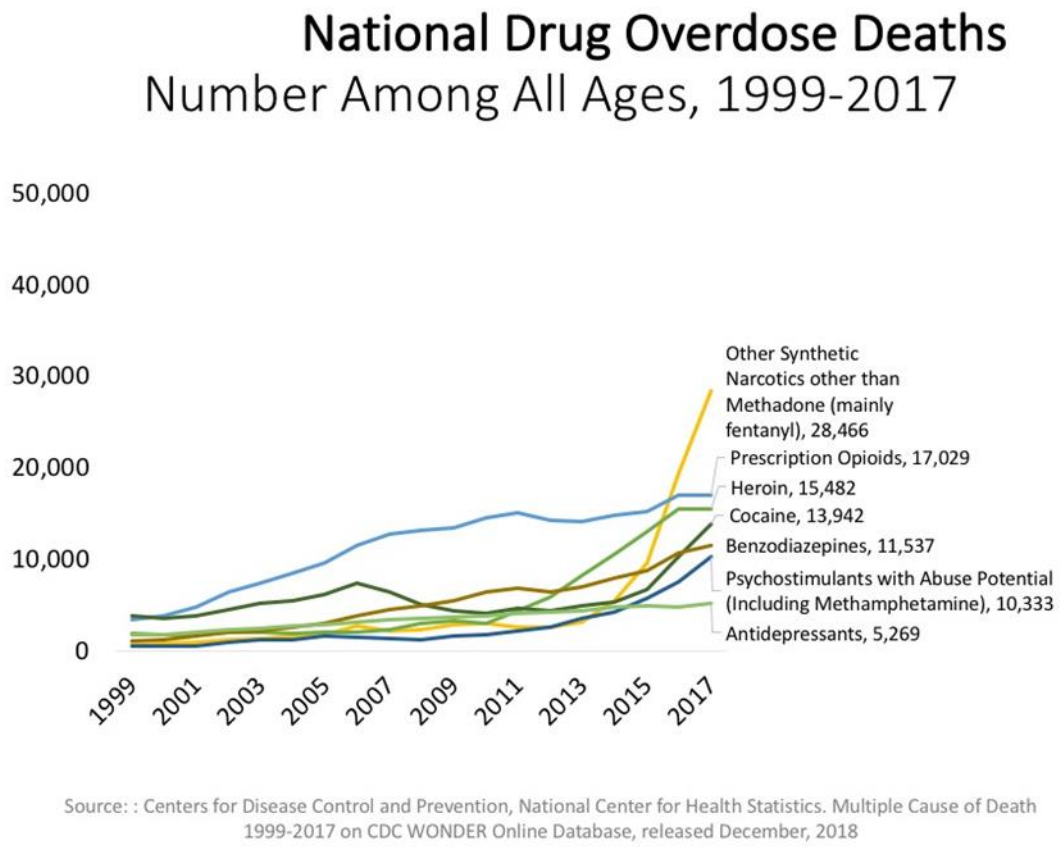


Figure 1: United States national drug overdose deaths; Number among all ages, 1999-2017. *Centers for disease control and prevention, National center for health statistics*

Substance use disorder is a worldwide issue. For instance, at this very moment, the USA is going through an "opioid crisis". In 2017, more than 47,000 Americans died as a result of an opioid overdose, including prescription or illicit opioids as illustrated in **Figure 1** (CDC/NCHS, 2018). States like Ohio, New-York and Florida are considerably more affected compare to others as 9, 6,7 and 6,8% of deaths by overdose occurred in each state respectively (Kaiser family foundation, 2017). That same year, an estimated 1.7 million people in the United States suffered from substance use disorders related to prescription opioid pain relievers, and 652,000 suffered from a heroin use disorder (Center for Behavioral Health Statistics and Quality, 2018). An European

opioid crisis seems rather limited mainly due to a stronger European regulation of opioids (van Amsterdam & van den Brink, 2015). This disastrous undergoing crisis highlights the need to better grasp the etiology of addiction and neurobiological changes induced by drugs of abuse. Right behind opioids, cocaine caused the death of almost 14000 people in the USA (**Figure 1**). Overall, national drug overdose deaths increased so dramatically (more that all the GIs deaths in Vietnam) that they decreased Americans citizens life expectancy (Los Angeles Times, 2017). In Europe, the precise numbers regarding cocaine overdoses are not available. Nonetheless, cocaine use prevalence increased dramatically in France; from 0.5 in 2000 to 3% in 2017 among the 18-34 years old population which raise strong concerns regarding death risks according to the European monitoring center for drugs and drug addiction (OEDT, 2019). Noteworthy, in the largest European cities such as Berlin, Bristol, Barcelona, Milan, Amsterdam, Paris, Zagreb, Lisbon and Brussels, the detection of benzoylecgonine (main cocaine metabolite, see below) is markedly increased in sewage (OEDT, 2019). Furthermore, law enforcements agents from European countries reported not only a record growth of cocaine seizures but also a higher cocaine purity in 2017 (OEDT, 2019).

Overall, these observations strongly suggest that the risks associated to cocaine use may increase overtime. Therefore, research towards finding a treatment for cocaine addiction and associated risks need more than ever a boost.

2. Cocaine

Cocaine (or methylbenzoylecgonine but also coke, booger sugar, snow, snuff, etc…) is a natural psychostimulant extracted from *Erythroxylon Coca*, the coca tree. Individuals usually use cocaine by intranasal inhalation under the powder form (or cocaine chlorohydrate). Cocaine is also smoked under its deprotonated form, crack cocaine (or “free base” cocaine). Originally, this plant grew naturally in Andes in south America where natives used to chew coca leaf to better tolerate altitude, cold and hunger. In 1752, Joseph de Jussieu brought the first specimen of coca tree in Europe that he discovered during his 36 years trip in South America. Almost one century later, the chemist Albert Niemann extracted and isolated cocaine from coca leaf in 1860. Over the years, cocaine use became popular in medicine for its analgesic properties but also in regular drinks. Indeed, Angelo Mariani created a very famous drink based on Bordeaux wine and cocaine named Mariani wine. The inventor of Coca-Cola, John Pemberton, might have copied the recipe to create his famous soda. In the 1900’s, cocaine addictive properties became clear and thus, the Harrison Act is the first law to regulate cocaine use in the USA in 1914. In Europe, cocaine is illicit (even if possession may be tolerated under 1 or 2 grams in several countries). Noteworthy, chewing of coca leaves does not induce addictive properties (Weil, 1981). However, their commercialization is still forbidden in most countries except south American countries such as

Peru and Bolivia which defend the therapeutic and nutritive properties of coca leaf (Duke *et al.*, 1975; Weil, 1981). To date, it's been 105 years since the first "cocaine law". However, understanding why individuals persist in consuming substances endangering their lives is still the central question.

i. Pharmacodynamic and metabolism of cocaine

Cocaine half-life is in between 40 to 60min in humans and then cocaine is mainly hydrolyzed by butyrylcholinesterase in liver and plasma (Nayak *et al.*, 1976; Javaid *et al.*, 1983). The main metabolites are benzoylecgonine and ecgonine methyl ester as illustrated in **Figure 2** (Zheng & Zhan, 2017). In lower proportions, Norcocaine, another metabolite, is formed upon cytochrome P450 action in the liver. Norcocaine reinforcing properties are similar to cocaine (Inaba, 1989). If cocaine use is combined with alcohol, another metabolite is formed, cocaethylene. Its clearance being slower (levels slightly above cocaine for a given period), alcohol prolonged cocaine effects (McCance *et al.*, 1995; Hart *et al.*, 2000).

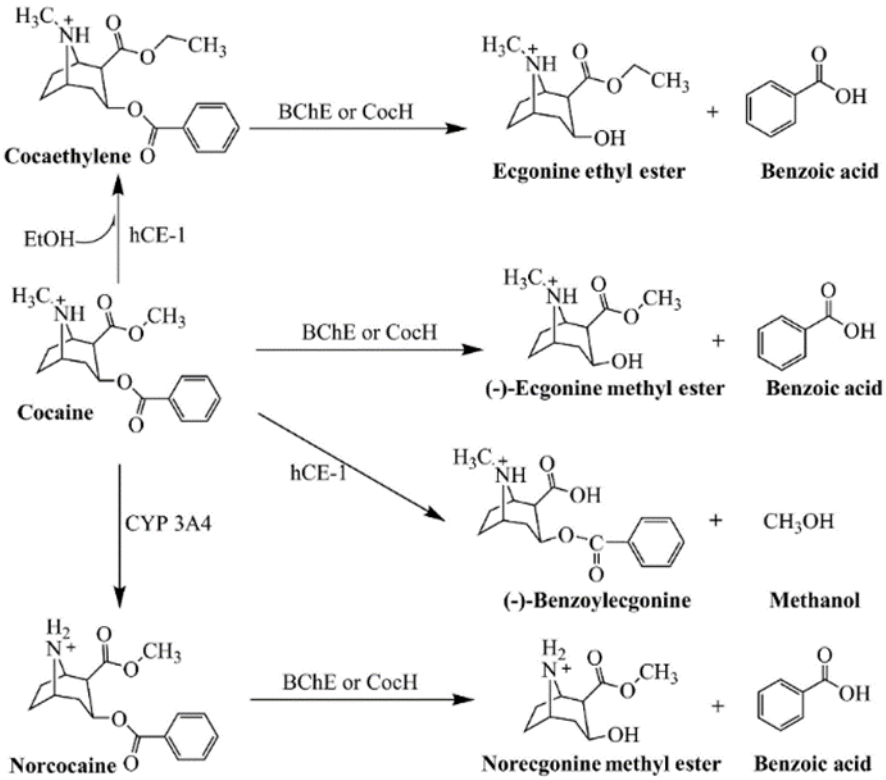


Figure 2: Metabolic pathways of cocaine. *Cocaine metabolites produced in humans through hydrolysis catalyzed by butyrylcholinesterase (BChE), oxidation by cytochrome P450 3A4 (CYP 3A4), and reaction of cocaine with ethanol (EtOH) (catalyzed by liver carboxylesterase 1 (hCE-1))* (Zheng & Zhan, 2017).

3. The reward system

To better understand why individuals with substance use disorder persist in consuming substances, research focused on the reward system of the central nervous system (CNS), which normally regulates motivation, emotions, memory and pleasure. This essential system is precisely the target of drugs of abuse.

In 1954, Olds and Milner stated for the first time the existence of reward centers and positive reinforcement in the CNS using electrical self-stimulation in rats (Olds & Milner, 1954). Several brain areas form the reward system gathered around the mesocorticolimbic dopaminergic pathway from the ventral tegmental area (VTA) to mainly the prefrontal cortex (PFC) and nucleus accumbens (NAc) **(Figure 3)**. Whether acting directly or indirectly on this system, all addictive drugs trigger dopamine release in the NAc and PFC from dopaminergic neurons in VTA.

Interestingly, the NAc can be divided into two sub-regions: NAc core and shell. NAc core controls learning and action during goal-directed behaviors (Carelli, 2004; Saddoris et al., 2013) whereas NAc shell is more involved in motivational processes and hedonic value (Kelley, 2004; Zorrilla & Koob, 2013; Castro *et al.*, 2015; Saddoris *et al.*, 2015).

The mesocorticolimbic dopaminergic pathway also sends projections to dorsal striatum (DS) and hippocampus (HPC) **(Figure 3)**. Interestingly, once drug taking is established, it is hypothesized that motivated behaviors towards drug intake usually encoded in NAc shift to the DS to become stimulus-response habits (compulsive use) (Hyman *et al.*, 2006). Regarding HPC, this area is involved in the learning processes linked to the drug associated context or cues, drug craving and relapse (Castilla-Ortega *et al.*, 2016; Kutlu & Gould, 2016; Gajewski *et al.*, 2017). The lateral habenula (LHb) inhibits the dopaminergic system via the GABAergic rostromedial tegmental nucleus (RMTg) **(Figure 3)**. Mameli's group showed that LHb contributes to aversive withdrawal symptoms (Meye *et al.*, 2015, 2016). Finally, the amygdala (Amy), composed of the basolateral nucleus (BLA) and the central nucleus (CeA), is also deeply involved in addiction. For instance, CeA is particularly involved in cocaine craving (Lu *et al.*, 2005, 2007). Overall, the reward system is a highly complex circuit as many structures are included in it and all of them are key players of addictive behaviors.

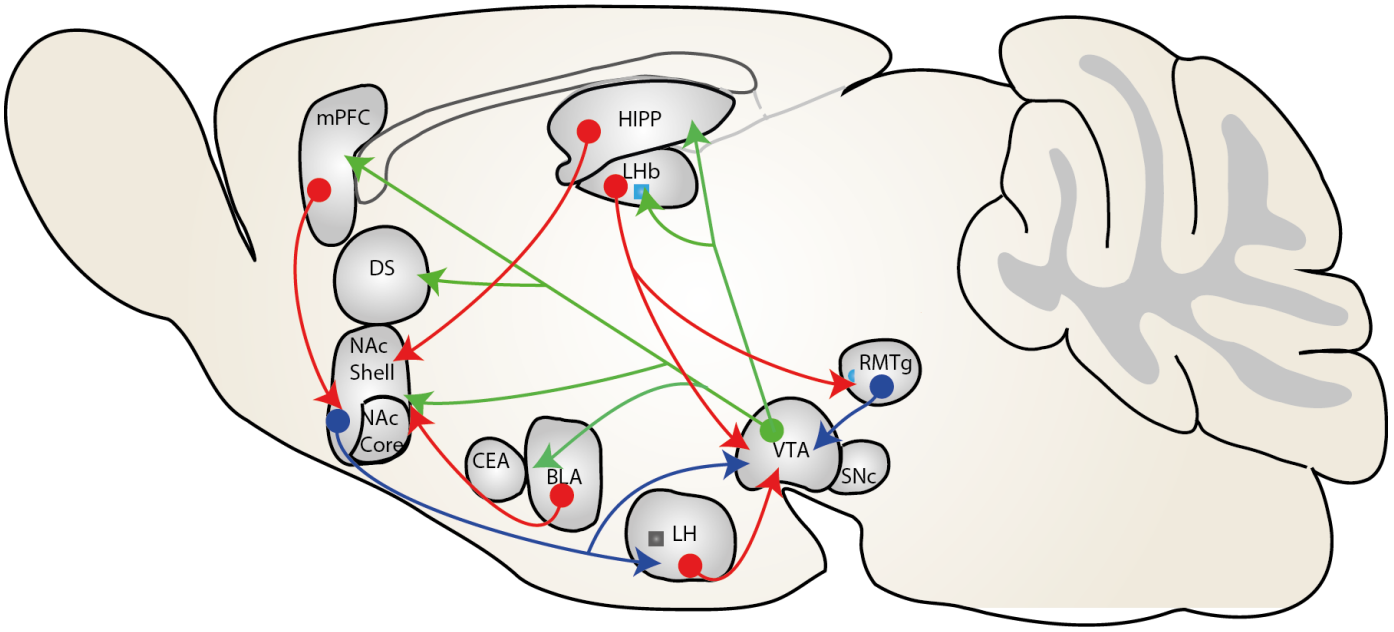


Figure 3: A simplified illustration of major dopaminergic, glutamatergic and GABAergic pathways in the rodent brain. *The reward circuit includes dopaminergic projections from the VTA to the NAc, which release dopamine in response to reward. There are also GABAergic projections from the NAc to the VTA. The NAc receives dense innervation from glutamatergic monosynaptic circuits from the PFC, HPC and Amy, among other regions. The VTA is also innervated by glutamatergic projections from Amy, LHb and lateral hypothalamus (LH), among others. Legend: dopaminergic = green; glutamatergic = red; GABAergic = blue. Adapted from (Russo & Nestler, 2013).*

ii. Dopamine

Dopamine is synthesized in dopaminergic neurons from the amino acid tyrosine. Tyrosine hydroxylase (TH) first catalyzes the transformation of tyrosine in L-DOPA. Then, L-DOPA turns into dopamine upon action of dopamine decarboxylase (Kopin, 1968). Next, dopamine is stored in synaptic vesicles through monoamine vesicle transporters (VMAT2). Upon release, dopamine is reuptaken through dopamine transporter (DAT) (Torres *et al.*, 2003). After reuptake, dopamine is either stored in vesicles or degraded by catechol-O-methyltransferase (COMT) and monoamine oxidase (MAO), resulting in the metabolite homovanillic acid (Eisenhofer *et al.*, 2004). Cocaine induces its psychostimulant effect by blocking DAT which results in an increase of DA levels and therefore enhanced stimulation of dopaminergic receptors associated with the feeling of euphoria in cocaine users (Volkow, Wang, Fischman, *et al.*, 1997).

iii. Serotonin

Serotonin (5-HT) neurons are located in raphe nuclei (brainstem) and innervate the whole CNS (Andén *et al.*, 1965). Notably, reward-related brain areas such as VTA, NAc, DS, PFC, Amy

and HPC are innervated by 5-HT pathways (Jacobs & Azmitia, 1992). 5-HT is synthetized from L-tryptophane upon catalyzation of tryptophane hydroxylase and 5-hydroxyptophane decarboxylase enzymes. 5-HT is then stored in synaptic vesicles through VMAT2 in the same way than dopamine (Eiden *et al.*, 2004). Similarly, 5-HT is also reuptaken by 5-HT transporter (SERT) then degraded by MAO. By blocking SERT, cocaine increases 5-HT levels in synapses which stimulates the 5-HT receptor family (Nichols & Nichols, 2008). Thus, many studies indicate that the serotoninergic system plays a major role in cocaine use (for review see (Filip *et al.*, 2010; Nonkes *et al.*, 2011)).

iv. Noradrenaline / Norepinephrine

Noradrenaline (in rodents) and norepinephrine (in humans) are synthesized from locus coeruleus which innervates forebrain, HPC, Hypothalamus (HYP), Amy and cerebellum (Zaniewska *et al.*, 2015). As well as 5-HT, norepinephrine is synthesized from dopamine by dopamine β-monooxygenase and then degraded by MAO or COMT. In the same way as dopamine and 5-HT, cocaine increases norepinephrine levels by blocking norepinephrine transporter (NET). Thus, the modulation of the noradrenergic/norepinephrine system also mediates cocaine effects (for review see (Sofuoglu & Sewell, 2009)).

4. Treatment strategies

Many clinical studies have been run to treat cocaine addicts over the years. However, there is no treatments being approved yet to treat cocaine addiction. Here, I summarize the findings over the years regarding the many different treatments tried to help individuals diagnosed with cocaine dependence. Therefore, I will describe here only the results from clinical trials and very briefly those from pre-clinical studies in some cases.

i. Dopamine

As dopamine signaling is severely altered in cocaine addict brain (Volkow, Wang, Fowler, *et al.*, 1997), therapeutic strategies were developed to activate D1 and/or D2 receptors. Unfortunately, treatments with D1 or D2 agonists produced poor or little therapeutic benefit for the management of cocaine use disorder (Haney *et al.*, 1998, 1999; Gorelick & Wilkins, 2006). I did not find many studies investigating a D2 antagonist strategy, probably due to the unwanted side-effects such as depressive-like behavior observed in humans (Loebl *et al.*, 2008) despite promising preclinical studies in squirrel monkeys (Bergman *et al.*, 1990; Spealman *et al.*, 1991). A D1 antagonist, Ecopipam, enhanced cocaine-SA in humans (Haney *et al.*, 2001). Another strategy was to block or enhance the reuptake or release of dopamine respectively. D-amphetamine and amphetamine isomers hold great promise, however, drugs such as Methylphenidate, Lisdexamfetamine (LDX) or Bupropion were ineffective to treat cocaine addicts (Stoops & Rush,

2013). Nonetheless, Bupropion seems to be interesting to treat nicotine dependence (Shiffman *et al.*, 2000).

ii. Serotonin

Although cocaine increases serotonin release, clinical trials using selective serotonin receptor ligands do not show promising effects (Johnson *et al.*, 2006; Winhusen *et al.*, 2014). As for dopamine, several selective serotonin reuptake inhibitors such as Fluoxetine (an antidepressant) have been tried in humans. However, Fluoxetine exhibited no significant effect on reducing the reinforcement properties of cocaine (Harris *et al.*, 2004; Winstanley *et al.*, 2011).

iii. Norepinephrine

Cocaine also elevates norepinephrine levels in brain. Using both agonist or adrenergic blocker, Sofuoglu et al. showed that adrenergic ligands did not alter subjective responses to cocaine (Sofuoglu *et al.*, 2000a, 2000b). However, propranolol, an adrenergic blocker, improved withdrawal symptoms in recovering cocaine addicts (Kampman *et al.*, 2001). More recently, Guanfacine (an α2A agonist) showed very promising results as this drug not only lowered stress and cue-induced cocaine craving but also enhanced inhibitory control and attentional shifting in cocaine addicts, with greater effects in women (Fox, Seo, *et al.*, 2012; Fox *et al.*, 2014, 2015; Moran-Santa Maria *et al.*, 2015).

iv. Acetylcholine

Cholinergic transmission is altered in both VTA and NAc of human and rodent brain (Sharkey *et al.*, 1988; Flynn *et al.*, 1992). Clinical studies using Varenicline, a partial α4β2 agonist, showed promising results as this drug reduced cocaine use and reward in cocaine users but further studies are still needed to confirm these findings and assess potential side-effects (Poling *et al.*, 2010; Plebani *et al.*, 2012). Biperiden, a cholinergic receptor blocker (mainly M1), diminished cocaine use and craving in cocaine addicts (Dieckmann *et al.*, 2014).

v. Glutamate

While chronic cocaine use increased glutamate release, withdrawal decreased glutamate levels in NAc (Baker *et al.*, 2003). The cystine/glutamate transporters play a major role in this as their stimulation prevented cocaine relapse and restored glutamate levels in rodents (Baker *et al.*, 2003). Thus, targeting cystine/glutamate transporters to treat cocaine addicts appeared as an interesting strategy. N-acetylcysteine stimulates glutamate release; therefore, several clinical studies explored its effects in cocaine users. Despite promising effects on cocaine use, N-acetylcysteine failed to exhibit robust effects on cocaine addicts across studies (LaRowe *et al.*, 2006, 2007, 2013; Mardikian *et al.*, 2007).

Metabotropic glutamate receptors represent a promising target as many studies indicate a role for glutamate metabotropic receptor 4, 5 and 7 in rodents (Li, Xi, *et al.*, 2013; Mao *et al.*, 2013;

Mihov & Hasler, 2016). Recent results from our consortium (ITMM CNRS) indicated a role for an orthosteric agonist for mGluR4 and 7 in reducing opiate reward (Hajasova *et al.*, 2018) as well as consumption of, motivation for and reacquisition of ethanol self-administration after abstinence (Lebourgeois *et al.*, 2018).

Our team investigated the effects of this same mGluR4 and mGluR7 agonist, the LSP29166, in a cocaine-SA paradigm with rats. Very interestingly, we observed an inhibition of the motivation towards cocaine (see *"**D**: Articles; **V**: Article 5: LSP29166, a novel orthosteric mGlu4 and mGlu7 receptor agonist, reduces cocaine self-administration under progressive ratio in rats").* However, there are no clinical trials regarding the relationships between glutamate metabotropic receptors and cocaine use to date.

vi. Targeting cocaine metabolism

As stated in previously, butyrylcholinesterase catalyze the hydrolysis of cocaine resulting in ecgonine methyl ester (or norecgonine methyl ester) and benzoic acid (Zheng & Zhan, 2017). Thus, accelerating cocaine metabolism to prevent its reinforcing effects appears as an ideal approach to treat not only cocaine relapse but also overdose (Zheng & Zhan, 2017). Mutant forms of human cocaine hydrolases are now under development to boost their catalytic activity and biological half-life before starting clinical trials (Chen *et al.*, 2016; Zheng & Zhan, 2017). In the same context, another strategy is to raise levels of butyrylcholinesterase permanently, using gene therapy (Murthy & Brimijoin, 2017).

vii. Motivational interviewing

Motivational interviewing is a psychotherapeutic approach used to enhance patient motivation towards a lifestyle change. It can rely on harsh techniques (a confrontation between the patient and therapist/family) or empathy in order to provoke/stimulate a development (White & Miller W. R., 2007; Miller & Rollnick, 2012). Thus, a motivational intervention reduces the risk of relapse in patients (Bernstein *et al.*, 2005). An analysis of 59 studies with a total of 13,342 participants with various substance use disorder concluded that motivational interviewing significantly reduces substance use in comparison with no intervention at all, but there is no difference compare to other psychotherapies (Smedslund *et al.*, 2011).

Mindfulness has shown benefits in individuals with a substance abuse disorder (Bowen *et al.*, 2009). Mindfulness is a therapy based on "paying attention in a particular way: on purpose, in the present moment, and non-judgmentally" (Bowen *et al.*, 2009). Very interestingly, a recent study demonstrated that a single Ketamine injection in combination with mindfulness-based relapse prevention diminished cocaine craving and risk to relapse in hulmans (Dakwar *et al.*, 2019).

viii. Vaccine therapy

Antibody targeting substances of abuse to inhibit their reinforcing properties in the central nervous system sounds like an innovative and elegant approach. However, giving the low molecular weight of cocaine, generating effective vaccines has been difficult. Only one anti-cocaine vaccine has completed clinical trials, the TA-CD vaccine comprised of succinyl norcocaine conjugated to cholera toxin B. Unfortunately, despite very promising results in phase 2, it failed to produce significant outcomes in phase 3 (Heekin *et al.*, 2017).

ix. Future directions

For decades now, research regarding treatments for cocaine addiction struggled to find an effective solution to help cocaine addicts, mainly for reducing the risk of relapse. According to the summary above, it appears that targeting specific receptors or transporters might not be the solution as these treatments either failed or produced side-effects. Interestingly, cannabidiol (CBD), a cannabinoid receptor 1 (CB1) and 2 (CB2) agonist, and CB2 agonists revealed promising results for reducing cocaine use in preclinical studies (Xi *et al.*, 2011; Zhang *et al.*, 2014; Luján *et al.*, 2018; Galaj *et al.*, 2019). Furthermore, targeting cocaine metabolism or preventing its reinforcing effects appears as a highly interesting approach as cocaine hydrolases are highly specific to cocaine and exhibit no side-effects. Future studies may highlight new approaches using allosteric modulators, receptor interacting protein or even gene therapy. For instance, several tools are currently available to edit epigenetic factors, like zinc finger proteins, transcription activator-like effectors, and the system of clustered regularly interspaced short palindromic Repeats (CRISPR) and CRISPR-associated protein 9 (Cas9) (Waryah *et al.*, 2018). Such approaches are highly promising as they provide accurate modification of a given type of epigenetic marks at a precise gene locus. Recently, a Korean team showed benefits in two mouse models of Alzheimer's disease using a CRSPR-Cas9 approach (Park *et al.*, 2019).

5. Vulnerability to cocaine addiction

i. Sex differences

Among American individuals using cocaine, around 17% develop cocaine dependence (Brady & Randall, 1999). Nonetheless, men are more prone to use cocaine due to impulsive behavior whereas women, displaying more likely anxiety and depressive-like behaviors, due to sociocultural factors as well as biological factors, are expected to relapse with greater risk (Becker & Hu, 2008; Becker *et al.*, 2012). However, even if women account for 56% of users at early ages (12-17 years old), this percentage seems to decrease overtime (Kerver & Becker, 2017). Moreover only 15% of users admitted in treatment in France were women in 2018 (Kerver & Becker, 2017; OEDT, 2019).

Very interestingly, these female/male differences are also observed in rodent models. First, female rats exhibit greater behavioral responses to both cocaine sensitization paradigm (Hu & Becker, 2003; Cummings *et al.*, 2014) and binge patterns of cocaine administration (Festa *et al.*, 2003) compare to male rats. These findings highlight estrogens (estradiol in rodents) as a cocaine sensitization enhancer. Indeed, estradiol increases escalation and motivation towards cocaine intake in female rats in cocaine-SA paradigms compare to males (Lynch *et al.*, 2001; Larson *et al.*, 2007; Perry *et al.*, 2013).

ii. Circadian rhythms

Daily rhythms are expressed in all organisms to synchronize themselves to the solar cycle. Internal oscillators (or circadian clocks) maintain this cycle around 24hr. One of these clocks is the hypothalamic suprachiasmatic nucleus (SCN) which is viewed as a master clock coordinating the activity of peripheral oscillator (Mendoza, 2019). Interestingly, individuals with disrupted circadian rhythms exhibit higher propensity for cocaine use (Mahoney *et al.*, 2014). Furthermore, chronic cocaine intake disrupts circadian activity both during cocaine intake and withdrawal in rodents (Stowie *et al.*, 2015). Indeed, chronic cocaine intake impacts the expression of circadian clock genes, such as *Per1*, *Per2*, *Per3*, *Bmal1* and *Clock*, which may participate in the vulnerability of individuals towards cocaine use (Wang *et al.*, 2019). Also, the LHb is viewed as another circadian clock influencing feeding but its role remains to be further detailed (Mendoza, 2019).

iii. Cocaine use in life

There are increasing evidences indicating drug use in elderly, in particularly in “baby boomers”. Very few studies have examined cocaine misuse in later life nor the specific adverse risks of cocaine use later in life (Yarnell *et al.*, 2019). Cocaine use could worsened neurologic, cognitive, and behavioral impairments in elderly people (Dokkedal-Silva *et al.*, 2018). Despite this recent trend, adolescents are more prone to consume cocaine and may develop a faster escalation intake (Spear, 2000). Even in rodents, adolescent rats also showed a more sensitive behavior towards cocaine use, as well as a greater escalation of cocaine intake (Wong *et al.*, 2013). Finally, prenatal exposure has been associated with increased likelihood of using both licit and illicit substances (Frank *et al.*, 2011) as well as attention deficit hyperactivity disorder, oppositional defiant disorder, depression and anxiety (Lambert & Bauer, 2012). Most studies regarding cocaine addiction in rodents choose to work with adolescent or young adults’ rats. Unfortunately, female physiology is not often studied in these studies. Thus, further studies are needed to better grasp the differences between females and males and investigating the vulnerability in elderly should not be set aside.

6. Animal models

Various animals are used to study cocaine effects in comparison with humans, such as zebrafish (Darland & Dowling, 2001), monkeys or rodents with the vast majority of studies being developed in rodents. Thus, rodent models rely on cocaine reward/reinforcement and motivation towards cocaine intake. To do so, studies classically run protocols such as behavioral sensitization, cocaine conditioned place preference (CPP) and cocaine self-administration (cocaine-SA) (Sanchis-Segura & Spanagel, 2006; Spanagel, 2017; Lamontagne & Olmstead, 2019).

i. Behavioral sensitization

The term sensitization refers to an increase of a response after the repeated occurrence of the stimulus that promoted the aforementioned response. Sensitization in drug abuse research has been mainly studied with respect to locomotor activity. Thus, the ability of addictive drugs to increase locomotion after an acute administration is progressively enhanced, when drug exposure is repeated. This so-called psychomotor sensitization is a very robust phenomenon that has been observed across rodents but not humans (Sanchis-Segura & Spanagel, 2006). Various protocols of behavioral sensitization exist, but briefly cocaine behavioral sensitization consists in either a single cocaine pre-exposure followed by another cocaine injection (Jackson & Nutt, 1993) or repeated cocaine injections followed by a withdrawal period or not (Blanco *et al.*, 2014). However, cocaine behavioral sensitization mainly reflects dopaminergic circuits sensitization which is not necessarily associated with motivation towards cocaine use (Robinson & Berridge, 2008).

ii. Cocaine conditioned place preference

CPP tests are performed in apparatus consisting of two or three compartment boxes in which animal associates a context A with cocaine (or other drugs) and a context B with a vehicle. Nonetheless, other paradigms rely on a single-compartment conditioning (Liu *et al.*, 2019). Thus, the conditioning relies on several factors such as the route of administration, time interval of drug administration, dose concentration, and the CPP apparatus used (Prus *et al.*, 2009). Usually, following repeated pairings of this drug/vehicle administration, on the test day, the animal will be allowed to freely move across compartments, usually under a drug-free state. An increased time spent in the drug-associated compartment is considered as a measure of conditioned preference (Sanchis-Segura & Spanagel, 2006). However, even if this procedure assesses cocaine reinforcement properties, cocaine CPP does not reflect the voluntary aspects of cocaine intake and therefore, may blunt modifications leading to cocaine addiction. For more insights regarding CPP see the review wrote by Tzschentke (Tzschentke, 2007).

iii. Cocaine self-administration

Cocaine-SA is viewed as the gold-standard procedure to assess cocaine-induced effects. This paradigm is based on an operant conditioning, meaning that animals must trigger an instrument (usually a lever or nose-poke) to obtain the drug. Thus, measuring the voluntary

cocaine intake reflects the reinforcement induced by cocaine. Therefore, the cocaine-SA model is closer to cocaine consumption in humans (Spanagel, 2017).

There are two different reinforcing protocols in such procedure: the "fixed ratio (FR)" and "progressive ratio (PR)". In a FR approach, the number of triggered responses to obtain cocaine is fixed all along cocaine-SA sessions. Thus, a FR1 or FR5 program means that the animal must trigger once or five times respectively, a lever or nose-poke to obtain the drug. The FR protocol is usually applied to investigate acquisition and maintenance of cocaine use (Caine & Koob, 1993). On the other hand, a PR approach consists in an exponential increase of the numbers of lever push or nose-poke to obtain the next cocaine dose. Thus, the "breaking point", defined as the highest response rate accomplished to obtain cocaine, reflects how far the animal works to obtain the reinforcer. Therefore, PR allows to investigate the animal motivation towards cocaine use (Arnold & Roberts, 1997).

Cocaine-SA also allows to model cocaine relapse by reinstating a cocaine-seeking behavior in animals following a period of withdrawal or extinction. Withdrawal is simply induced by putting the animal in his home cage for a variable period (usually few weeks to a month) while during an extinction paradigm, the animal is tested under the same operant conditions of cocaine-SA without access to the drug. Extinction is completed when operant responding appears to be extinguished (Spanagel, 2017). One can measure reinstatement at that point which is triggered by either a stress exposure, cocaine injection or re-exposure to cocaine-associated cues (Shaham *et al.*, 2003). In more complex cocaine-SA models using punishment following cocaine intake (such as electrical foot shocks), only 15 to 20% or rats self-administer cocaine during a 3-month period (Deroche-Gamonet *et al.*, 2004; Vanderschuren & Everitt, 2004).

As stated previously, cocaine-SA appears as the gold-standard method to model cocaine addiction. As this disease is highly complex, it makes sense to use this model in order to assess the many modifications triggered by cocaine in brain.

II. Cocaine addiction: a focus on molecular adaptations

Before I describe the molecular adaptations occurring following cocaine intake, I briefly introduce the main described cellular alterations induced by cocaine.

1. Cellular modifications

Giving the many cocaine-induced effects on brain cellular plasticity, the purpose of this thesis is not to give an exhaustive review of these adaptations as they have been previously described (Thomas & Malenka, 2003; Maze & Russo, 2010; Russo *et al.*, 2010; Zhou *et al.*, 2014). Thus, I will here focus only on the cellular adaptations helping the understanding of our work.

i. Morphological alterations

Cocaine increases spine dendritic density in key areas of the mesocorticolimbic system such as NAc and CPF (Robinson & Kolb, 1999a, 2004; Robinson *et al.*, 2001). Interestingly, in transgenic mice with cocaine-insensitive DAT, increased dendritic spine density in the NAc is absent suggesting that these morphological changes are dopamine-dependent (Martin *et al.*, 2011). Similarly, prenatal cocaine exposure alters cortical and hippocampal neurons in adulthood which results in cognitive impairments in both preclinical models and humans (Thompson *et al.*, 2009). Frontal lobes are associated to prenatal exposure processes and interestingly, cocaine-exposed children showed a greater activation in frontal cortex and caudate compare to controls in a fMRI study (Sheinkopf *et al.*, 2009).

ii. Hippocampus functional changes

HPC undergoes strong alteration in terms of activity upon cocaine use. Showing cocaine-associated cues to cocaine dependent individuals triggered an increased HPC activation (Grant *et al.*, 1996; Prisciandaro *et al.*, 2014). This was also observed in awake rats upon cocaine intake (Febo *et al.*, 2005). In rodents, cocaine-SA enhanced long-term potentiation (LTP) at CA1 synapses (del Olmo *et al.*, 2006) and this effect was persistent until 10 days after cocaine exposure (Thompson *et al.*, 2004). These changes were associated with enhanced spines density in CA1 of Lewis but not F344 rats following cocaine-SA (Miguéns *et al.*, 2015), probably due to the many physiological differences in response to drugs of abuse (Rivera, Miguéns, *et al.*, 2013). In combination, AMPA and NMDA receptor expression increases in post synaptic density following cocaine-SA but this effect is directly abolished after 1 day of withdrawal in CA1 (Caffino *et al.*, 2014). Giving these findings, one could wonder whether cocaine affects learning. Interestingly, after 22 days of cocaine-SA, Del Olmo et al. assessed whether cocaine facilitates spatial learning in a highly demanding water maze task (four sessions, two trials per session with a 90-min intertrial interval). Very interestingly, cocaine enhanced memory of the platform location (Del Olmo *et al.*, 2007) as well as in a Y-maze task in Lewis rats (Fole *et al.*, 2017). It should be notified that the same group did not find any changes in a previous study using a protocol lasting 3 days, with three consecutive trials per day and an intertrial interval of just 1 min (Del Olmo *et al.*, 2006). Thus, cocaine enhances animal performance only in high demanding task.

Understanding cocaine-induced cellular adaptations is of great interest as they enable to better grasp the physiological consequences upon cocaine use. However, to shed light on these adaptations, one must understand the molecular origin of these alterations.

2. Molecular modifications specific to the nucleus accumbens

Giving the crucial role of NAc in reward, many studies investigated the molecular adaptations occurring in this site upon passive or voluntary cocaine intake.

i. Transcription factors

Transcription factors are proteins able to bind a specific DNA sequence. Noteworthy, cocaine modulates several transcription factors which then modulate the transcription of a battery of genes that could explain cellular adaptations above. Here, I will summarize the main transcription factors regulated upon cocaine intake:

- cAMP response element-binding protein (CREB): cocaine increased CREB expression in NAc and VTA. In turn, CREB altered cocaine reinforcing properties in both mice and rats (Carlezon *et al.*, 1998; Olson *et al.*, 2005).
- AP-1 (FosB & JUN dimer): cocaine elevated FosB expression in striatum (Couceyro *et al.*, 1994; Hope *et al.*, 1994; Nye *et al.*, 1995) as well as JUN in both striatum and midbrain of rats and humans (Couceyro *et al.*, 1994; Moratalla *et al.*, 1996; Bannon *et al.*, 2014).
- EGR family (early growth response): a considerable amount of studies demonstrated the induction of EGR1 (Bhat *et al.*, 1992a; Bhat & Baraban, 1993; Helton *et al.*, 1993; Daunais & McGinty, 1994, 1995; Ennulat *et al.*, 1994; Drago *et al.*, 1996; Hammer & Cooke, 1996; Yuferov *et al.*, 2003; Lynch *et al.*, 2008; Piechota *et al.*, 2010), EGR2 (Yuferov *et al.*, 2003; Piechota *et al.*, 2010), EGR3 (Morris *et al.*, 1998; Chandra *et al.*, 2015) and EGR4 (Piechota *et al.*, 2010) by cocaine in striatum of rodents.
- NFκB (nuclear factor kappa-light-chain-enhancer of activated B cells): cocaine abuse up-regulated NFκB levels in striatum and midbrain of rodents and humans (Ang *et al.*, 2001; Russo *et al.*, 2009; Bannon *et al.*, 2014). In turn, NFκB inhibition blocked cocaine reinforcing effects in rodents (Russo *et al.*, 2009).
- Glucocorticoid receptor (GR): cocaine decreased GR expression levels in human cells (Malaplate-Armand *et al.*, 2005). Interestingly, GR inactivation in mice decreased cocaine sensitization, CPP and self-administration (Ambroggi *et al.*, 2009; Barik *et al.*, 2010). Furthermore, administration of a GR antagonist dose-dependently reduced the motivation to self-administer cocaine (Deroche-Gamonet *et al.*, 2003).

Obviously, these cocaine-induced changes of transcription factors expression strongly implicate that cocaine alters gene expression. For an exhaustive review regarding the effects of cocaine on transcription factors, see (Gonzalez-Nunez & Rodríguez, 2017).

ii. Transcriptional adaptations

A large number of studies investigated cocaine-induced transcriptional adaptations in brain using either candidate gene or genome-wide approaches. Regarding the candidate gene approach, results have been reviewed (Lull *et al.*, 2008; Chen *et al.*, 2009). Moreover, we performed this kind of approach by focusing on the endocannabinoid system (ECS) and opioid system (OS) coding-genes following cocaine intake. Therefore, I give a more exhaustive description of the literature regarding how cocaine modulates both systems in sections *"**D**: Articles; **I**: cocaine-induced neuroadaptations of the endocannabinoid system in reward-related brain regions: new insights into epigenetic regulations of cannabinoid genes"* and *"**D**: Articles; **II**: voluntary cocaine intake modulates mu opioid receptors in the hippocampus"*.

Tools such as microarrays and RNA sequencing (RNA-Seq) were then developed to analyze transcriptional adaptations on a genome-wide scale. Such approaches allowed the analysis of broader changes and even gene networks alterations. Results using these approaches following cocaine intake have been described in detail (Bannon *et al.*, 2005; Hemby, 2010). In our recent review, we went through the various results obtained with this type of approach, highlighting critical factors for transcriptome analysis in addiction, and in particular in cocaine addiction, such as animal models, paradigms, cell diversity and sex differences (De Sa Nogueira *et al.*, 2019) (see below and see figure 1 of the review).

Transcriptomic technologies rapidly evolved to the first single-cell RNA-Seq reported (Tang *et al.*, 2009). To do so, several cell or nuclei separating methods exist, the easiest and cheapest being flow-cytometry (Stark *et al.*, 2019). Thus, as cell heterogeneity can cause serious variability in the results, this technology will improve the understanding of transcriptomic alterations in the CNS. Overall, this approach answers to new biological questions and give a more detailed picture of the changes one can observe in the brain (for review see (Stark *et al.*, 2019)).

Some argue that false positive could still occur using single-cell technology (Lacar *et al.*, 2016). Therefore, several studies went even further by performing single-nuclei RNA-Seq, an even more powerful technology reducing the likelihood of aberrant transcription, resulting in a whole transcriptome sequencing from a single nucleus (Grindberg *et al.*, 2013). This powerful tool is useful in heterogeneous tissues such as the HPC. For instance, Lacar et al. performed a single-nuclei RNA-Seq from individual activated dentate granule neurons to assess transcriptional patterns associated with induction of activity (Lacar *et al.*, 2016).

3. Neuroepigenetics and addictive behaviors: where do we stand?

In 1957, the term epigenetic was introduced as the idea that identical genotypes can give rise to different phenotypes (Waddington, 1957). All epigenetic mechanisms occur in neurons,

which gave rise to the study of neuroepigenetics. Thus, this term describes the study of the epigenetic mechanisms such as DNA methylation (DNAm), histone modifications and non-coding RNAs (ncRNAs) occurring in the CNS. All these different mechanisms control gene expression without altering the DNA sequence. By the end of the 2000's, the analysis of the epigenome, or whole epigenetic changes occurring in one organism, quickly rises to better explain these molecular adaptations. To date, a vast number of studies investigated whether cocaine intake modulates transcription of accumbal genes as reviewed by Nestler (Nestler, 2014). Indeed, there is abundant research regarding the link between addictive behaviors and neuroepigenetics. As our group is highly interested in these processes, Katia Befort, Karine Merienne (a collaborator and expert in neuroepigenetics in our institute) and I wrote a review regarding this question entitled "Neuroepigenetics and addictive behaviors: Where do we stand?" published in *Neuroscience Biobehavioral Reviews* in 2019. In this review available in **Annex 1**, we discuss the advances and limits of genome-wide neuroepigenetics, or epigenome, regarding psychostimulants, cannabinoids, opioids and alcohol. We therefore reviewed the mechanisms of DNAm and posttranslational modifications of histones. Following the addition of a methyl group by DNA methyl transferases (DNMT) at cytosine-phosphate-guanine (CpG) dinucleotides, gene expression is often associated with gene expression decrease when DNAm occurs in gene promoter. Regarding histones, several modifications can occur at the N-terminal part of histones tail such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation. Depending on the histone tail and amino acid, these alterations can up- or down-regulate gene expression. Powerful genome wide analysis tools, such as chromatin or methylated DNA immunoprecipitation followed by sequencing (and others), were created to better capture the relationships between the epigenome and cocaine (see **Box** in (De Sa Nogueira *et al.*, 2019)).

Among ncRNAs, microRNAs (miRNAs) are well characterized for their role in the modulation of translation. miRNAs are small (∼22 nucleotides) RNA transcripts that do not code for a given protein, but function by repressing target mRNA(s) translation by binding to complementary targets. Giving their small size, each miRNA can potentially target hundreds to thousands of mRNA transcripts (Jonkman & Kenny, 2013). The major role of miRNAs in the reinforcing properties of cocaine have been previously reviewed (Kenny, 2014).

For a detailed overview of genome wide studies investigating the effects of drugs of abuse such as psychostimulants, opiates, alcohol, cannabinoids and nicotine, see our review "Neuroepigenetics and addictive behaviors: Where do we stand?" in Annex 1.

4. Epigenetic factors: a focus on HDAC and Mecp2

Among the very first indications of epigenetic regulations induced by cocaine, Jean Zwiller's team was precursor in exploring the role of two proteins involved in histone acetylation and methylation processes and their role regarding cocaine addiction: the methylated DNA-binding protein 2 (MeCP2) and the histone deacetylase 2 (HDAC2).

i. MeCP2

MeCP2 binds methylated DNA to recruits transcriptional regulators. It has been associated with Rett syndrome, a severe neurodevelopmental disorder (Amir *et al.*, 1999). Chronic cocaine treatment and cocaine-SA increased MeCP2 expression in reward-related areas such as PFC, DS and HPC in rats (Cassel *et al.*, 2006; Host *et al.*, 2011; Pol Bodetto *et al.*, 2014). Other groups proposed that MeCP2 expression level in striatum may be necessary to convey the effects of cocaine in rats (Im *et al.*, 2010). Interestingly, downregulation of MeCP2 in NAc increased amphetamine-CPP (Deng *et al.*, 2010). Clearly, more studies are needed to better clarify the role of MeCP2 in addictive behaviors.

ii. HDAC2

A wide number of studies described histone acetylation modifications in response to drugs of abuse. For instance, cocaine increases H3 and H4 acetylation in NAc (Kumar *et al.*, 2005). Zwiller's group showed that cocaine-SA increased HDAC2 gene expression in rat striatum (Host *et al.*, 2011). Using HDAC modulators, one can easily observe the effect of acetylation or deacetylation on cocaine intake. For instance, HDAC inhibitors decreased cocaine intake, motivation towards cocaine intake and cocaine seeking (Romieu *et al.*, 2008, 2011) in rats, indicating that behavior can be modulated by epigenetic factors. However, as many studies investigated the role of HDAC inhibitors in cocaine intake, I describe further these results in section *"**E**: Discussion; **3**: Epigenetic prospects"*. Altogether, the role of HDAC2 in cocaine is getting well described. However, whether specific genes are targeted by HDAC2 remain to be determined.

III. Binge-eating disorder: is it food addiction?

1. General aspects

i. Definition

First described in the 50's, binge eating disorder (BED) got a special attention these last 15 years (**Figure 4**). BED episodes are characterized by a huge amount of food intake within a short period

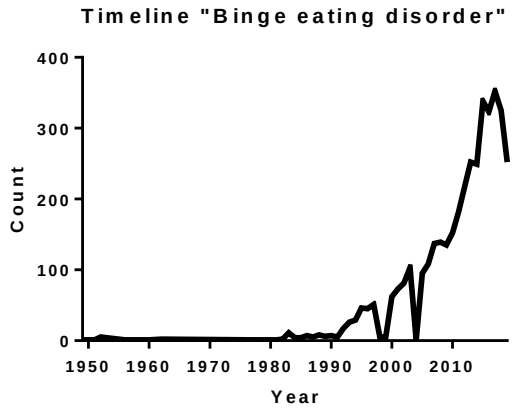


Figure 4: timeline of publications regarding "binge eating disorders" in PubMed.

of time. Several criteria have been established to diagnose someone as an individual with BED (**Table 1**). One should note that weight is not part of the diagnostic criteria for binge eating disorder (**Table 1**)**.** In DSM5, BED severity is characterized as mild, moderate, severe or extreme according to the number of episodes in a week (see level of severity, **Table 1**). BED is often confused with bulimia nervosa (BN), another eating disorder characterized by binge eating, but the major difference lies in the compensatory behaviors (such as purging, exercise, etc…) that are present in BN and absent in BED (American Psychiatric Association, 2013).

Table 1: Binge eating disorder diagnostic criteria from DSM5

Criterion	
A	Recurrent episodes of binge eating. An episode of binge eating is characterized by both of the following: -1: Eating, in a discrete period of time (e.g., within any 2-hour period), an amount of food that is definitely larger than what most people would eat in a similar period of time under similar circumstances. -2: A sense of lack of control overeating during the episode (e.g., a feeling that one cannot stop eating or control what or how much one is eating)
B	The binge-eating episodes are associated with three (or more) of the following: -1: Eating much more rapidly than normal -2: Eating until feeling uncomfortably full -3: Eating large amounts of food when not feeling physically hungry -4: Eating alone because of feeling embarrassed by how much one is eating -5: Feeling disgusted with oneself, depressed, or very guilty afterward
C	Marked distress regarding binge eating is present
D	The binge eating occurs, on average, at least once a week for 3 months
E	The binge eating is not associated with the recurrent use of inappropriate compensatory behavior as in bulimia nervosa and does not occur exclusively during the course of bulimia nervosa or anorexia nervosa

Level of severity	
Mild	1–3 binge-eating episodes per week
Moderate	4–7 binge-eating episodes per week
Severe	8–13 binge-eating episodes per week
Extreme	14 or more binge-eating episodes per week

From Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition, 2013 American Psychiatric Association

ii. Sex differences and vulnerability

BED lifetime prevalence is 2.9% in women and 3.0% in men (Hudson *et al.*, 2012) and the majority of individuals with BED are either overweight or obese (de Zwaan, 2001). Compare to other eating disorders such as anorexia nervosa (AN) or BN in which the vast majority of afflicted individuals are women, the prevalence in BED is more even (Bulik *et al.*, 2015). Nevertheless, among women with BED, prevalence of major depression and anxiety disorders is higher and family histories of BED is greater compare to obese women without BED as controls (Fowler & Bulik, 1997; Javaras *et al.*, 2008). As BED aggregate within families, its heritability was found to be close to 50% in twin studies (Javaras *et al.*, 2008; Mitchell *et al.*, 2010). Furthermore, an adoption study showed that environmental factors do not play a major role in BED whereas genetic factors are highly involved highlighting the importance of genetic factors in BED (Klump *et al.*, 2009).

In the same context, candidate-gene association studies found single-nucleotide polymorphisms (SNPs) in humans or animals' models of BED. Interestingly, SNPs occurred in genes such as *Mc4r*, *Drd2*, *Ghrelin* and *Oprm1* (for review see **Table 1** in (Bulik *et al.*, 2015)), all genes involved in reward and food intake. Recently, the first genome-wide association study regarding binge-eating behavior (associated to bipolar disorder) found SNPs in *PRR5* and *ARHGAP8* genes which respectively encode a subunit of mTORC2 and a protein from the RhoGAP family (McElroy *et al.*, 2018) but further studies are needed to define their role in BED. Overall, little is known about the genetics factors leading to, or induced by, BED. Genome-wide studies appeared as a powerful tool to better uncover which genes are involved in BED and their function.

iii. Vulnerability influenced by circadian rhythms

As with cocaine, mutations within circadian genes disrupt circadian rhythm and alters feeding. For instance, homozygous *Clock* (a central mediator of circadian rhythm) mutant mice are hyperphagic and obese (Turek *et al.*, 2005). Mutations within *Per2* abolishes anticipation of mealtime in mice (Feillet *et al.*, 2006) as well as palatable food intake (Bainier *et al.*, 2017). Furthermore, *Rev-Erbα* mutant mice exhibit a far more important sucrose intake when sucrose is reintroduced following deprivation (Feillet *et al.*, 2017). Finally, several SNPs found in *Clock* and *Per2*, have been associated with obesity in humans (Lopez-Minguez *et al.*, 2016). Altogether, examples above highlight the critical role of circadian genes in hedonic feeding and strongly suggest their involvement in the vulnerability towards eating disorders. For further data regarding the relationships between circadian rhythm, food and palatable food intake, see the review from Mendoza (Mendoza, 2019).

iv. Treatments

The only drug approved by the US food and drug administration to treat BED is LDX. LDX is an inactive prodrug promoting the release of monoamines (McElroy *et al.*, 2015, 2016; Gasior *et al.*, 2017). Among other drugs with proven efficacy on BED but not yet approved:

- Memantine (NMDA antagonist) reduced binge episodes frequency in humans (Hermanussen & Tresguerres, 2005; Brennan *et al.*, 2008)
- Fluoxetine reduced binge-eating in rodents (Cifani *et al.*, 2009)
- Duloxetine and Sibutramine (SERT inhibitor) reduced both the frequency of binge episodes crisis, body weight, and depressive symptoms in patients with BED (Appolinario *et al.*, 2003; Milano *et al.*, 2005; Guerdjikova *et al.*, 2012).
- Atomoxetine (norepinephrine reuptake inhibitor) reduced binge episodes frequency in humans (McElroy *et al.*, 2007)
- Zonisamide and Citalopram (anti-convulsant) reduced binge-eating, weight loss and binge episodes frequency in humans (McElroy *et al.*, 2003; Ricca *et al.*, 2009)

However, most of these drugs also produce adverse effects. As BED diagnosis is still quite recent, further studies will probably find new and better treatments regarding BED. For more data regarding BED treatment, see the following reviews (Cuesto *et al.*, 2017; Himmerich & Treasure, 2018).

2. Involvement of the reward system

Before going further, it is essential to grasp the difference between homeostatic and non-homeostatic feeding (the latter also being named hedonic feeding). Thereafter, I introduce the cerebral systems involved in these processes.

i. Homeostatic and non-homeostatic feeding

While the homeostatic pathway controls energy balance by increasing the motivation to eat following depletion of energy store, non-homeostatic or hedonic processes increase the desire to eat palatable food despite energy abundance and regardless of satiety (Lutter & Nestler, 2009). The literature regarding food intake is abundant. Therefore, I will gradually describe, but not exhaustively, the pathways involved in food intake. First, research on homeostatic control focused mostly on circulating hormones that relay information about peripheral energy levels to the brain. Two of the most important peripheral hormones are leptin and ghrelin **(Figure 5)**. Leptin is synthesized by white adipose tissue and suppresses food intake and stimulates metabolic processes to dissipate excessive energy stores (Lutter & Nestler, 2009). In contrast, ghrelin is a stomach-derived peptide whose level increases in response to negative energy balance and stimulates food intake and energy storage (Lutter & Nestler, 2009). Once transported in the circulatory system, both hormones act on brain structures among which, the HYP appears as one of the most important brain structures for feeding. For a more complete description of the actual literature, see the recent review from Rossi & Stuber (Rossi & Stuber, 2018).

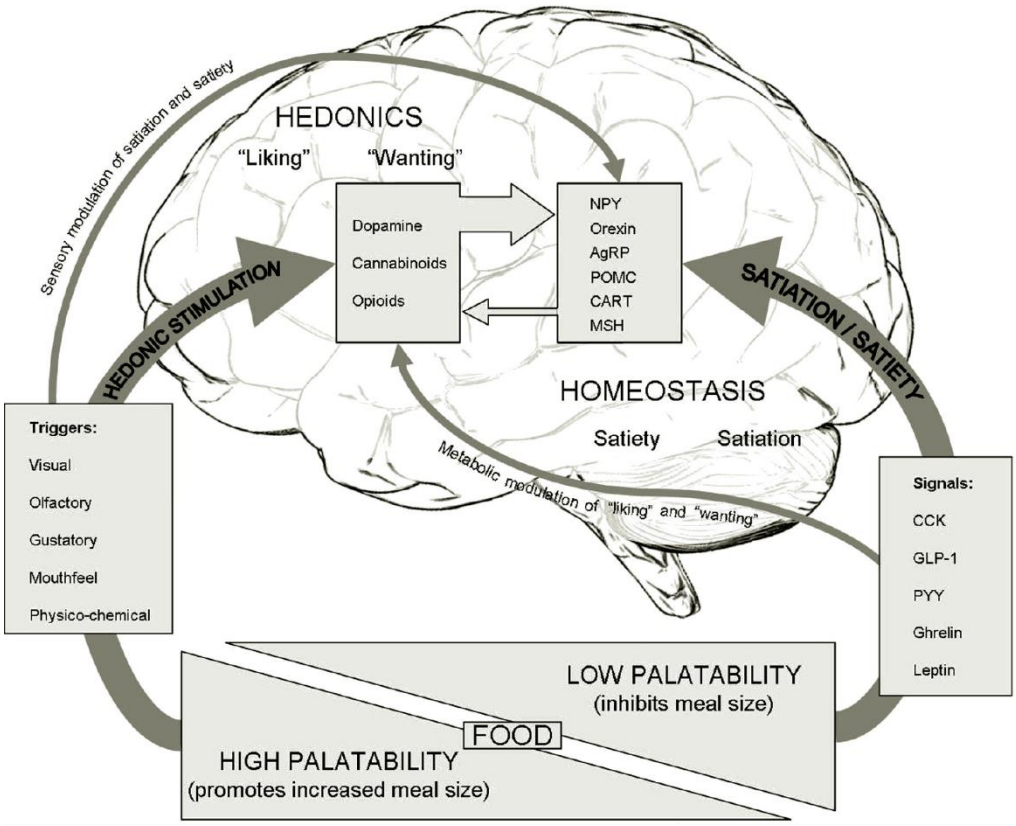


Figure 5: schematic illustration interaction between brain homeostatic and hedonic pathways. *Palatable food stimuli trigger hedonic stimulation which activate the dopaminergic, endocannabinoid and opioid system, resulting in a promotion of food intake. On the other hand, satiety signals interact with same system but lead to an inhibition of food intake. From (Gibbons & Blundell, 2015).*

ii. Arcuate nucleus of the hypothalamus

Leptin and ghrelin receptors are expressed in the arcuate nucleus (Arc), a HYP sub-region. Briefly, two subsets of neuron populations have been identified: pro-opiomelanocortin (POMC) and cocaine-amphetamine-regulated transcript (CART) neurons versus neuropeptide Y (NPY) and agouti-related peptide (AgRP) neurons. POMC/CART neurons are associated with food intake suppression upon leptin activation while leptin inhibits NPY/AgRP neurons, which increases food intake. On the opposite, NPY/AgRP neurons express ghrelin receptors which, upon activation, promote food intake (Lutter & Nestler, 2009). More recently, two other neuron populations with the ability to drive food intake have been found in Arc. Upon stimulation, TH neurons promote food intake (Zhang & van den Pol, 2016) while ARC neurons expressing oxytocin receptor induce satiety (Fenselau *et al.*, 2017). Yet further work is needed to elucidate the connectivity of these neurons' subsets.

iii. Dopaminergic system

The dopaminergic system has been described as an essential system for feeding. For instance, lesion of dopaminergic neurons by 6-hydroxydopamine induces aphagia (Ungerstedt, 1971) and, accordingly, invalidation of TH in dopaminergic neurons in mice produces the same effect (Zhou & Palmiter, 1995). Furthermore, optogenetic activation of VTA GABAergic neurons, which reduces dopamine release, inhibits licking for sucrose in mice (van Zessen *et al.*, 2012). Very interestingly, the dopaminergic is also the target of both leptin and ghrelin. Indeed, leptin inhibits DA neurons within VTA (Fulton *et al.*, 2006) which decreases DA release in NAc in rats (Krügel *et al.*, 2003). In contrast, ghrelin stimulates DA neurons activity which produces a DA increase in NAc of rodents (Naleid *et al.*, 2005; Abizaid *et al.*, 2006; Jerlhag *et al.*, 2007). Thus, similar to what has been hypothesized for drugs of abuse, chronic, repeated stimulations of dopaminergic system in the NAc by palatable food and associated cues shifts signaling to dorso-striatal dopaminergic pathways resulting in habit formation (Everitt & Robbins, 2016). Therefore, compulsive eating is thought to reflect a maladaptive stimulus-driven habit, which overrides voluntary, goal-directed actions (Moore *et al.*, 2017). The involvement of the reward system in both feeding and binge-eating is further described in the following reviews (Lutter & Nestler, 2009; Moore *et al.*, 2017; Liu & Kanoski, 2018; Rossi & Stuber, 2018).

iv. Is sugar addiction real: comparisons between sugar addiction in humans and animal models

Over the years and following the increased number of studies regarding palatable food intake, sugar appeared as a danger for health. Indeed, clinical trials and epidemiologic studies have shown that individuals who consumed higher amounts of added sugar, tend to gain more weight and have a higher risk of obesity, type 2 diabetes mellitus, dyslipidemias, hypertension and cardiovascular disease (Yang *et al.*, 2014). Despite knowing that, 75% of the US population consumes more than 10% of calories from added sugars (Yang *et al.*, 2014). Dr Avena argue that if people persist consuming despite adverse consequences, this is probably due to addictive-like behavior due to food/sugar addiction. On the other hand, others argue there is still little evidence supporting sugar addiction. Indeed, according to Westwater et al. still little is known in humans regarding the reward system implication in sugar addiction. Even if most of evidences come from animal models, they argue that these models still relies on limited-access which do not reflect human intake of sugar (Westwater *et al.*, 2016). I describe further this scientific debate in the discussion of this thesis.

3. Animals models of binge eating

Several models are available to study eating disorders and understand their etiology. Over the years, different diets such as the well-known cafeteria diet (chocolate, peanut butter, sweetened condensed milk, etc…) have been developed for animal models. However, these types of diet do not necessarily reflect binge-eating. Indeed, they are often presented to the animals *ad libitum*. Therefore, models with limited access to palatable food or food-restriction were then developed overtime displaying overeating within a short period of time, the main BED criteria. Here, I briefly give a description of these models allowing to investigate BED.

i. Limited access models

Avena et al. described a model with intermittent and limited access to a sugar solution (10% sucrose) and chow 12hr daily, followed by 12hr of deprivation, which lasts for 4 weeks. As rats typically feed at the onset of the dark, sucrose/chow intake start 4hr after the onset of the dark cycle and the onset of food access cycle and thus rats will be hungry when food is presented (Avena et al., 2006). In this paradigm, rats drink 20% of their total daily sugar intake within the first hour of access (Avena *et al.*, 2006), which reflects binge-eating. Corwin et al. described a model using pure solid vegetable fat. Briefly, rats have a 2hr access to fat every day for 4 weeks while a side-group have access only 3 days a week. Interestingly, the side-group binged more on fat compare to the non-restraint group (Corwin *et al.*, 1998; Corwin, 2004). In that model, animals are not food restricted compare to the binge-sucrose model above.

ii. Stress-induced hyperphagia model

In female rats exposed to multiple food restriction and refeeding cycles, an acute foot-shock as stress factor induced binge-like intake of palatable food (Hagan *et al.*, 2002). Instead of using foot-shock to induce stress, maternal separated animals (model of stressful experience in early life) also exhibited binge-eating when exposed to restriction/feeding cycles (Jahng, 2011).

iii. Sham-feeding model

This model involves a gastric fistula to induce drainage of consumed food before it enters the intestine. Thus, rats show an increase in binge eating behavior (Davis & Campbell, 1973). However, even if this model reflects binge-like eating behavior, it is unsuitable to investigate BED but rather BN. Indeed, individuals with BED do not exhibit compensatory behaviors (such as purging) as stated above.

iv. Genetic models

Mutations in the *Leptin* gene are very well known for inducing obesity in humans as well as in mice ($Lep^{ob/ob}$) which also exhibit hyperphagia (Montague *et al.*, 1997; Clément *et al.*, 1998). Furthermore, invalidation of critical genes for feeding in mice such as *Pomc*, *Mc3r*, or *Mc4r* induced hyperphagia and obesity as well (Fan *et al.*, 1997; Huszar *et al.*, 1997; Yaswen *et al.*, 1999; Butler *et al.*, 2000). Interestingly, hyperphagia is also induced in 5-HT2C-KO mice and selective restoration of 5-HT2C in POMC neurons in 5-HT2C-KO mice rescued hyperphagia (Tecott *et al.*, 1995; Xu *et al.*, 2008). However, it should be mentioned that even if BED mouse models are also obese, and although there is a significant comorbidity between obesity and BED, not all patients with BED are obese as stated previously. For more data regarding genetic models and eating disorders, see the exhaustive review and **Table 2** from Bulik et al. available below (Bulik *et al.*, 2015).

Gene	Description	Feeding	Body weight	Adiposity
Pmch	Pro-melanin-concentrating hormone	↓	↓	↓
Chrm3	Cholinergic receptor, muscarinic 3	↓	↓	↓
Cnr1	Cannabinoid receptor 1	↓	↓	↓
Oprd1	Opioid receptor delta 1	↔	↔	↔
		Resistant to HFD	Resistant to HFD	Resistant to HFD
Lep	Leptin	↑	↑	↑
Lepr	Leptin receptor	↑	↑	↑
Pomc	Proopiomelanocortin	↑	↑	↑
Mc3r	Melanocortin 3 receptor	↔	↔	↑
Mc4r	Melanocortin 4 receptor	↑	↑	↑
Nmu	Neuromedin U	↑	↑	↑
Htr2c	Serotonin receptor 2C	↑	↑	↑
Htr1b	Serotonin receptor 1B	↑	↑	↔
		Males only		

Table 2: Eating disorder phenotypes in KO-mouse models. *These findings reflect phenotypes under ad libitum feeding conditions unless otherwise specified. ↑ indicates increase in phenotype, ↓ indicates decrease in phenotype, whereas ↔ indicates no change in phenotype. Abbreviation: HFD (high fat diet). From (Bulik et al., 2015).*

v. Environment affects food addiction

Many studies investigated whether environment factors can be linked to eating disorders. Clinicians associate individuals with BED to many factors such as parenting, life distress, weight teasing, western media promoting thinness, etc…(Mazzeo & Bulik, 2009). Most studies exploring the link between eating disorder and epigenetic focused on DNAm, in particular in individuals with AN. Interestingly, these studies found DNAm changes at genes promoter such as *DRD2*, *DAT*, *Pomc* and *Cnr1* in blood from patients with AN (Bulik *et al.*, 2015). As research exploring epigenetics changes in eating disorder is rather recent, these findings must be carefully interpreted. Indeed, these results come from blood samples, therefore one should avoid

generalization suggesting similar modifications in brain structures. However, animal models of eating disorder should help towards the understanding of epigenetic modifications in that matter. Further studies should explore these changes in BED or BN models.

4. Common neurobiological basis of drug and food addiction

The shift from impulsive palatable food intake towards compulsive eating behavior quickly raised the question whether these responses account for "food/palatable food addiction". First, there is an overlap between brain structures involved in drug and natural food craving. For instance, when showing either palatable food or chocolate to cravers from these foods during fMRI, studies observed an enhanced activity in HPC, insula, caudate, orbitofrontal cortex, NAc and cingulate cortex (Pelchat *et al.*, 2004; Rolls & McCabe, 2007). All these structures have been described as involved in drug craving (Koob & Volkow, 2010; Everitt & Robbins, 2016). There are also similarities regarding D2 availability between obesity and addictive behaviors. It is very well known that D2 decreased availability is either the cause (Everitt *et al.*, 2008) or consequence (Volkow *et al.*, 1999; Nader *et al.*, 2002) of addictive behavior. Interestingly, D2 is also decreased in obese subjects (Volkow *et al.*, 2008). Furthermore, the opioid system also plays a major role in both hedonic feeding and drugs intake. For instance, Mu opioid receptor (MOP) modulate opioid, ethanol, nicotine and psychostimulants mediated reward (Contet *et al.*, 2004; Trigo *et al.*, 2010; Charbogne *et al.*, 2014). Similarly, Naloxone, a MOP antagonist, attenuated appetite in individuals with eating disorder (Drewnowski *et al.*, 1995). Dr. Avena identified many commonalities between drugs of abuse intake and the binge sucrose model. Overtime, many studies from her group identified behavioral and neurochemical commonalities between this model and drug abuse which are summarized in **Table 3** (Bocarsly & Avena, 2013). This work brings important and new insights towards bridging food and drugs of abuse addiction.

Substance dependence parameters	**Animal model of sugar dependence**
Behavioral signs	
Tolerance	Escalation of daily sugar intake (Colantuoni *et al.*, 2001)
Signs of withdrawal	Somatic signs (teeth-chattering, tremor) Anxiety (measured by plus-maze) Ultrasonic distress vocalizations (Colantuoni *et al.*, 2002; Avena, Bocarsly, *et al.*, 2008)
Consuming more than intended	Deprivation effect (Avena *et al.*, 2005)
Locomotor cross-sensitization	Amphetamine (Avena & Hoebel, 2003)

Proclivity to consume other drugs of abuse	Alcohol (Avena *et al.*, 2004)
Neurochemical changes in NAc	
Repeated release of dopamine	(Rada *et al.*, 2005; M. Avena *et al.*, 2006)
↑ D1 receptor binding	(Colantuoni *et al.*, 2001)
↓ D2 receptor binding	(Colantuoni *et al.*, 2001)
↑ D3 receptor mRNA	(Spangler *et al.*, 2004)
↑ preproenkephalin mRNA	(Spangler *et al.*, 2004)
Dopamine/Acetylcholine imbalance during withdrawal	(Colantuoni *et al.*, 2002; Avena, Bocarsly, *et al.*, 2008)

From (Bocarsly & Avena, 2013)

Table 3: summary of findings in support of sugar addiction in rats using an animal model of sucrose or glucose bingeing.

IV. The endocannabinoid system

1. Description of the ECS

i. Origin and discovery

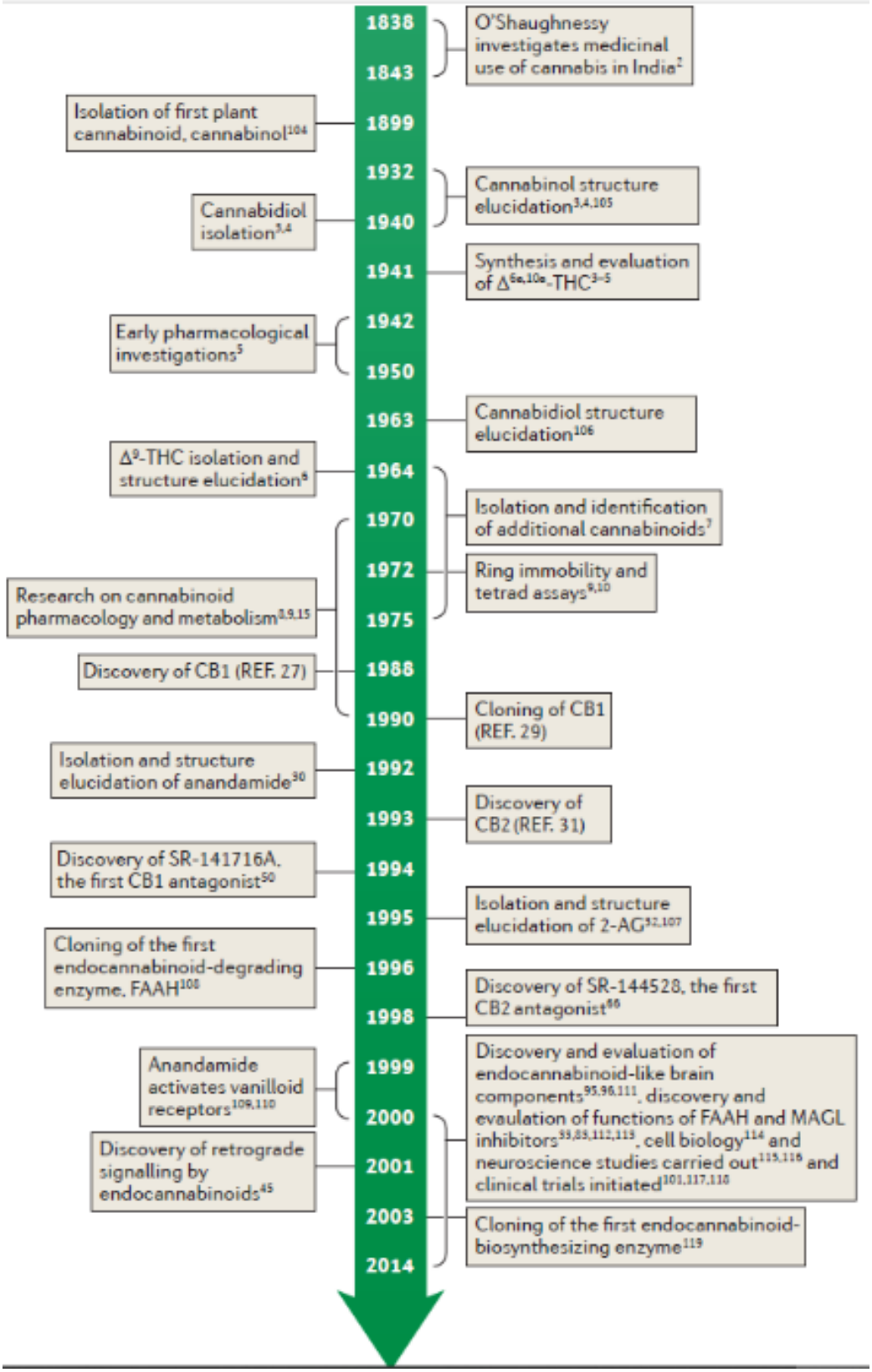
To date, more than 150 million people regularly smoke cannabis, making it one of the world's most popular recreational drugs of abuse (Lawler, 2019). In France, around 20% among the 15-34 years old population consumed cannabis at least once in 2018 making this country with the highest percentage of consumers in Europe (OEDT, 2019). Even though the origin of cannabis is not clear, *Cannabis Sativa* has been used for many years in ancient China for medicine (Mechoulam *et al.*, 2014). Recent archeological findings indicated use of cannabis in religious activities or rituals at least 2500 years ago (Ren *et al.*, 2019). At the beginning of the 19th century, an Irish physician William Brooke O'Shaughnessy observed the effects of *Cannabis Sativa* on tetanus and other convulsive diseases in India and then introduced it in British medicine in Europe (O'Shaughnessy, 1843). Despite the medicinal potential of *Cannabis Sativa* (**Figure 6**), the USA banned the plant from the pharmacopeia by establishing the "Marijuana Tax Act" in 1937. Unfortunately, this contributed to the decrease of scientific interest to further explore cannabis properties and it promoted its demonization. In France, Cannabis is illegal since 1925 (Convention de Genève / Convention de l'ONU 1961).

The structure of the main component of *Cannabis Sativa,* the Δ9-tetrahydrocannabinol (THC), was discovered in 1964 (Gaoni & Mechoulam, 1964). Many years later followed the discovery of its main receptor, CB1, in rat brain (Devane *et al.*, 1988). The development of molecular biology approaches then allowed the cloning of the gene coding for this receptor, *Cnr1* (Matsuda *et al.*,

1990). Recently, the crystal structure of CB1 has been reported (Hua *et al.*, 2016; Shao *et al.*, 2016), which bring new opportunities to target CB1 even more effectively. Indeed, increasing the knowledge of CB1 3D structure will allow not only to create new selective ligands but also to discover new protein interactions. Several years later, CB2, another cannabinoid receptor, was identified in periphery (Munro *et al.*, 1993). CB1 and CB2 presence in vertebrates can be traced back to the puffer fish, birds and amphibians (Elphick & Egertová, 2005) and the role of the ECS is highly conserved across species (Elphick, 2012). Both cannabinoid receptors are $G\alpha_{i/o}$ protein coupled receptors (Glass & Northup, 1999) belonging to the rhodopsin-like subfamily, composed of seven transmembrane helices and interconnected by 3 intracellular and 3 extracellular loops (Shim, 2010). Both CB1 and CB2 share 48% of amino-acids sequence homology (Howlett *et al.*, 2002). Another potential cannabinoid receptor has been described, GPR55 (also named cannabinoid receptor 3). GPR55 is also coupled to a $G_{i/o}$ protein (Ryberg *et al.*, 2007; Lauckner *et al.*, 2008) and interacts with cannabinoid ligands (Pertwee *et al.*, 2010). GPR55 share 13 and 14% amino acid sequence homology with CB1 and CB2 respectively (Sawzdargo et al., 1999). However, its classification as a cannabinoid receptor is still debated as it also binds Lysophosphatidylinositol (Hurst *et al.*, 2017).

Arachidonoylethanolamide or Anandamide (AEA) (which means bliss/happiness in Sanskrit), was the first endocannabinoid (eCB) discovered (Devane *et al.*, 1992), followed by the identification of 2-arachidonoylglycerol (2-AG) 3 years later (Mechoulam *et al.*, 1995). The discovery of the ECS increased the scientific interest for a better understanding of its functional properties. It is now clear that this system is far more complex than initially thought of and its physiological role is still not fully understood yet.

***Figure 6: Timeline representing cannabis and endocannabinoid research*.** From (Mechoulam *et al.*, 2014).



2. Ligand binding to CB1, CB2 and GPR55

Several cannabinoid ligands exist: eCBs (endogenous cannabinoid ligands), phytocannabinoids (cannabinoids which originate from plants) and synthetic cannabinoids (artificial cannabinoids). All cannabinoid ligands bind to cannabinoid receptors, with distinct affinities, see **Table 4** (Pertwee *et al.*, 2010).

Ligand	Ki CB1 (nM)	Ki CB2 (nM)	Reference
Agonists with similar affinity at CB1 and CB2			
Δ^9-THC	5.05–80.3	3.13–75.3	(Pertwee, 2008)
HU-210	0.06–0.73	0.17–0.52	(Pertwee, 2005)
CP55940	0.5–5.0	0.69–2.8	(Pertwee, 2005)
WIN55212	1.89–123	0.28–16.2	(Pertwee, 2005)
Anandamide	61–543	279–1940	(Pertwee, 2005)
2-AG	58.3, 472	145, 1400	(Pertwee, 2005)
CB1 selective agonists			
ACEA	1.4, 5.29	195 >2000	(Pertwee, 2005)
Arachidonylcyclopropylamide	2.2	715	(Pertwee, 2005)
Methanandamide	17.9–28.3	815–868	(Pertwee, 2005)
Noladin ether	21.2	>3000	(Pertwee, 2005)
CB2 selective agonists			
JWH-133	677	3.4	(Pertwee, 2005)
HU-308	>10000	22.7	(Pertwee, 2005)
JWH-015	383	13.8	(Pertwee, 2005)
AM1241	280	3.4	(Pertwee, 2005)
CB1 selective antagonists			
Rimonabant (SR141716A)	1.8–12.3	514–13,200	(Pertwee, 2005)
AM251	7.49	2290	(Pertwee, 2005)
AM281	12	4200	(Pertwee, 2005)
LYS320135	141	14,900	(Pertwee, 2005)
Taranabant	0.13, 0.27	170, 310	(Fong *et al.*, 2007)
NESS 0327	0.00035	21	(Pertwee, 2005)
O-2050	2.5, 1.7	1.5	(Martin, Stephenson, *et al.*, 2002)
CB2 selective antagonists			
SR144528	50.3–>10,000	0.28–5.6	(Pertwee, 2005)
AM630	5152	31.2	(Pertwee, 2005)
JTE-907	2370	35.9	(Pertwee, 2005)
Other compounds			
11-OH-Δ^8-THC	25.8	7.4	(Pertwee, 2005)
Ajulemic acid	5.7, 32.3	56.1, 170.5	(Dyson *et al.*, 2005; Pertwee, 2005)

Cannabinol	120–1130	96–301	(Pertwee, 2008)
Cannabigerol	81	2600	(Cascio *et al.*, 2010)
Cannabidiol	4350–>10,000	2399–>10,000	(Pertwee, 2008)
***N*-Arachidonoyl dopamine**	250	12,000	(Bisogno *et al.*, 2000)
Virodhamine	912	N.D.	(Steffens *et al.*, 2005)

LEGEND

Phytocannabinoid

Endocannabinoid

Synthetic cannabinoid

Table 4: Ki values of CB1 & CB2 receptor ligands. *Values obtained by in vitro displacement of a tritiated compound from specific binding sites on rat, mouse, or human CB1 and CB2 receptors. Adapted from (Pertwee et al., 2010). 11-OH-Δ^8-THC is not highlighted in color as this compound is formed in the body after decarboxylated cannabis is consumed (Kraemer & Paul, 2007).*

AEA and 2-AG are CB1 and CB2 agonists with similar affinity for CB1 and CB2. THC and WIN55.212 exhibit a higher affinity compare to eCBs. Overtime, specific CB1 and CB2 agonists have been synthetized such as Arachidonyl-2'-chloroethylamide (specific CB1 agonist) and (6aR,10aR)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro -6,6,9-trimethyl-6H-dibenzo[b,d]pyran (JWH133) (specific CB2 agonist). Cannabidiol exhibit a very low affinity for both CB1 and CB2. Cannabidiol may also be an antagonist of GPR55 (Ryberg *et al.*, 2007). Overall, these compounds enabled a more precise understanding of the ECS and revolutionized the study of the ECS.

3. Cannabinoid receptor signaling

i. CB1 signaling

As mentioned previously, CB1 is a member of the $G\alpha_{i/o}$ protein coupled receptor family and its signaling pathway is rather complex. CB1 activation inhibits forskolin-stimulated adenylyl cyclase by coupling to the pertussis toxin -sensitive $G\alpha_{i/o}$. Therefore, cAMP is decreased and also, G-protein-coupled inwardly rectifying potassium channels are activated while N-type and P/Q type voltage-gated calcium channels are inhibited (Howlett *et al.*, 2002; Pertwee, 2006a; Turu & Hunyady, 2010; Howlett & Abood, 2017; Ye *et al.*, 2019). All these processes lead to an inhibition of the presynaptic terminal. Furthermore, stimulation of CB1 also leads to phosphorylation and activation of mitogen-activated protein kinases and increases the phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 through G-protein dependent and β-arrestin 1/2 dependent pathways (Flores-Otero *et al.*, 2014; Delgado-Peraza *et al.*, 2016). Noteworthy, reports mentioned coupling to $G_{q/11}$ in human embryonic kidney 293 cells and cultured hippocampal neurons (Lauckner *et al.*, 2005) as well as coupling with $G\alpha_s$ in Chinese hamster ovary cells (Glass & Felder, 1997; Abadji *et al.*, 2008), human embryonic kidney 293 cells (D'Antona *et al.*, 2006; Finlay *et al.*, 2017) and cultured striatal medium spiny neurons (Laprairie *et al.*, 2014). Very interestingly, Araque's lab demonstrated multiple times that astroglial CB1 activation in HPC elevates calcium

level in astrocytes, therefore providing further evidence towards a coupling with $G_{q/11}$ (Navarrete & Araque, 2008, 2010; Robin *et al.*, 2018). Even a coupling with G_z has been reported (Garzón *et al.*, 2009).

ii. CB2 signaling

To date, research indicates that CB2 couples preferentially to $G_{\alpha i}$ (Glass & Northup, 1999; Bash *et al.*, 2003; Garzón *et al.*, 2009). Thus, upon CB2 activation, the same intracellular pathways described above for CB1 are induced. However, very recent data indicate that, upon CB2 activation, M-type potassium currents are enhanced (Ma *et al.*, 2019).

iii. Biased signaling at cannabinoid receptor

Biased signaling is the concept that different ligands acting on the same G protein-coupled receptor (GPCR), in the same tissue, can give rise to markedly different cellular responses (Ibsen *et al.*, 2017). For instance, the primary divergence in signaling occurs at the selection between pathways generated via G proteins, versus pathways generated by β-arrestins **(Figure 7)** (Howlett & Abood, 2017). Targeting the ECS without adverse effects would be very promising for clinical studies, thus, understanding the molecular actions of cannabinoid receptor ligands is a priority. To clarify these processes, I here only describe endocannabinoids-induced bias signaling at CB1 and CB2.

Upon CB1 activation, 2-AG was one of the more potent mediators for β-arrestin 2 recruitment compare to other cannabinoids. Moreover, both eCBs enhanced $G\alpha_{i/o}$ signaling. Interestingly, 2-AG and AEA treatment lead to increased CB1 gene and protein expression in a striatal progenitor cells line (Laprairie *et al.*, 2014). 2-AG also elicited prolonged dwell times and promoted short-term G protein and longer-term β-arrestin signaling (Flores-Otero *et al.*, 2014). In another study, AEA was biased towards cAMP inhibition compare to 2-AG (Khajehali *et al.*, 2015). Thus, despite some similarities, eCBs exhibit a biased signaling at CB1. Therefore, studies measuring eCBs levels and/or CB1 expression must take these eCBs elicited pathways in consideration. Regarding CB2, AEA showed a balanced outcome across assays, but 2-AG was prominently biased towards the β-arrestin pathway compared to G-protein signaling (Soethoudt *et al.*, 2017). In another study, 2-AG displayed higher potency for ERK signaling activation but inhibited cAMP production at higher concentrations (Shoemaker *et al.*, 2005). For review of cannabinoid bias signaling at CB2 see Morales et al. (Morales *et al.*, 2018).

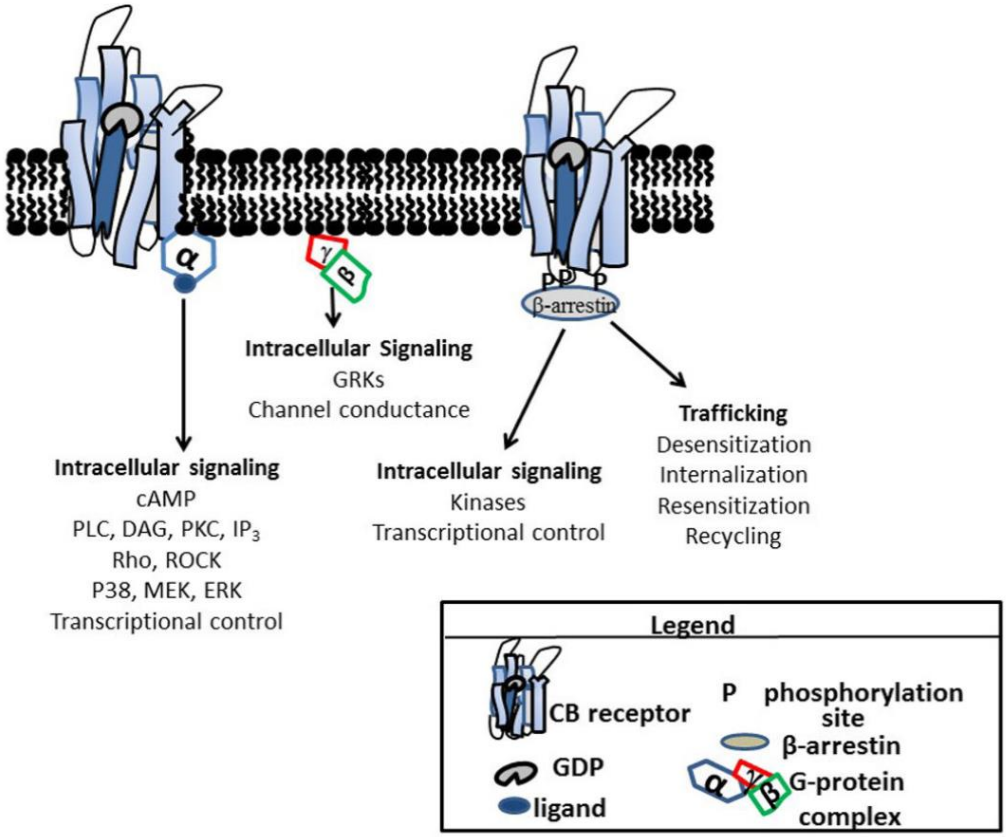


***Figure 7: biased agonism of cannabinoid receptor**. Phosphorylation of the receptor recruits β-arrestin, which, in addition to directing internalization, can also initiate intracellular signaling. From (Howlett & Abood, 2017).*

4. Unusual cannabinoid receptor interacting proteins

As any GPCRs, activation of CB1 phosphorylates Serine/Threonine residues generally in the C-terminal tail or third intracellular loop, by one of several G-protein-coupled receptor kinases **(Figure 7)**. Then, the phosphorylated-CB1 recruits the cytoplasmic proteins β-arrestin ½ **(Figure 7)** (Smith *et al.*, 2010). CB1 also interacts with GPCR-associated sorting protein which mediate endocytic sorting. A report showed an interaction between CB1 and Src homology 3-domain growth factor receptor-bound 2-like (endophilin) interacting protein 1 (SGIP1). SGIP1 was discovered in 2005 and its overexpression in mice lead to obesity (Trevaskis *et al.*, 2005). Upon interaction, SGIP1 prevents the endocytosis of activated CB1 and maintain its expression at the membrane. Moreover, SGIP1 alters CB1 signaling in a biased manner as ERK 1/2 pathway is negatively regulated by SGIP1 (Hájková *et al.*, 2016).

Another interacting protein has been described for CB1: the cannabinoid receptor-interacting protein, with two forms CRIP1A and CRIPB encoded by the *Cnrip* gene. Discovered in 2007 (Niehaus *et al.*, 2007), CRIP1A was described as the form interacting with CB1 (and not CB2) which

attenuates the constitutive CB1 mediated inhibition of voltage-dependent Ca^{2+} channels. Noteworthy, CRIP1a did not alter CB1 expression (Niehaus *et al.*, 2007). Nevertheless, a subsequent report knocked-down CB1 expression in rat striatum, which was associated with a concomitant increase of both mRNA and protein CRIP1A expression (Blume *et al.*, 2013). Recently, several reports demonstrated that CRIP1A modulate further CB1 signaling. In heterologous models, CRIP1a over-expression reduced basal phospho-ERK levels (Blume *et al.*, 2015), enhanced CB1-mediated G-protein activation and attenuated CB1 downregulation (Smith *et al.*, 2015). Interestingly, CRIP1a overexpression attenuated both depolarization-induced suppression of excitation and inhibition elicited by cannabinoid in cultured hippocampal neurons (Smith *et al.*, 2015). *In vivo*, hippocampal CRIP1a overexpression enhanced CB1 activity (Guggenhuber *et al.*, 2016). To date, CRIP1A role in behavior has never been investigated although a thesis entitled "Functional characterization of CRIP1a knockout mice" by Joanna Jacob in 2009 is available on semantic scholar (www.semanticscholar.org). In this manuscript, she confirms that CRIP1A and CB1 expressions are independent in CRIP1A-KO mice, indicating that CRIP1A does not affect CB1 expression. Surprisingly, CB1 activity was only affected in amygdala in which she observed a cannabinoid-elicited enhancement associated with anxiolytic-like phenotypes in CRIP1A-KO mice. Unfortunately, these findings are not published. Therefore, these results should be carefully viewed. Thus, research on SGIP1 and CRIP1A impact on ECS physiological functions in brain still deserves more attention. To date, no further data regarding CRIPB function is available.

5. Cannabinoid receptors heterodimerization

Many studies point out the heteromerization of CB1, a physical association with another GPCR leading to functional interactions, such as CB1-CB2 heteromerization (Derouiche & Massotte, 2019). Indeed, CB1 forms functional heteromers with CB2 which produces a heteromer print (bidirectional cross-antagonism) (Callén *et al.*, 2012; Navarro *et al.*, 2018). Studies also suggested a CB1-MOP heteromerization as electron microscopy reports showed colocalization of both receptors in striatum (Rodriguez *et al.*, 2001; Pickel *et al.*, 2004). Another report suggested a functional interaction between CB1 and MOP in co-transfected cells (Human embryonic kidney 293, Neuro-2A and human neuroblastoma cells) (Rios *et al.*, 2006). Several studies described a CB1-D2 heteromerization in cultured primary striatal neurons. Interestingly, this heteromer could induce opposite effects, such as a stimulation of adenylate cyclase, contrary to the effect upon activation of the individual receptors (Glass & Felder, 1997; Jarrahian *et al.*, 2003; Kearn *et al.*, 2005; Marcellino *et al.*, 2008). A study using electron microscopy showed overlapping subcellular distributions of CB1 and D2 immunoreactivities in NAc of rat providing further evidence for their heteromerization (Pickel *et al.*, 2006). Another study in co-transfected cells showed that CB1 is

interacting with the D2L form of D2 (instead of D2S) but also CB1 and D2L form heterotetramers consisting of CB1 and D2L homodimers (Bagher *et al.*, 2017).

Other reports either demonstrate or suggest CB1 heteromerization with other GPCR both in heterologous systems and *in vivo*:

- CB1 and delta opioid receptor in Neuro2A transfected cells (Rozenfeld *et al.*, 2012) or rat cortex (Bushlin *et al.*, 2012)
- CB1 and AT1 receptor in transfected human embryonic kidney 293 cells (Rozenfeld *et al.*, 2011)
- CB1 and A2A receptor in transfected human neuroblastoma cell (Carriba *et al.*, 2007)
- CB1 and 5-HT2A receptor in transfected human embryonic kidney 293 cells (Viñals *et al.*, 2015)
- CB1 and GPR55 receptor in rat and monkey striatum (Martínez-Pinilla *et al.*, 2014)
- CB1 and OX1 receptor in transfected human embryonic kidney 293 cells (Ellis *et al.*, 2006; Ward *et al.*, 2011)

Reports also mentioned CB2 heteromers complexes. In a model of hypoxia-ischemia brain, the authors observed an up-regulation of CB2 and 5-HT1A complexes (Franco *et al.*, 2019). In cultured microglial cells, a study observed that CB2 forms heteromer complex with GPR18 (and not CB1 with GPR18), which also produced a bidirectional cross-antagonism phenomenon (Reyes-Resina *et al.*, 2018). Apart from the CNS, CB2 interacts with HER2 or GPR55 in breast cancer cells (Moreno *et al.*, 2014; Blasco-Benito *et al.*, 2019). In human embryonic kidney 293 cells, CB2 also interacts with GPR55 which altered signaling of both receptor (Balenga *et al.*, 2014).These recent discoveries hold great promises as this complex could be targeted in antitumor therapies. Overall, these findings open a new frontier of research towards targeting specific neuron populations or modulating specific behavior with higher accuracy (Ferré *et al.*, 2009).

6. CB1 distribution in brain

CB1 is the most expressed GPCR in the brain and its distribution is now well characterized both in rodent (Herkenham *et al.*, 1991; Tsou *et al.*, 1998) and human (Westlake *et al.*, 1994). CB1 is highly expressed in brain structures like globus pallidus, HPC, substantia nigra reticulata and cerebellum as illustrated in **Figure 8**.

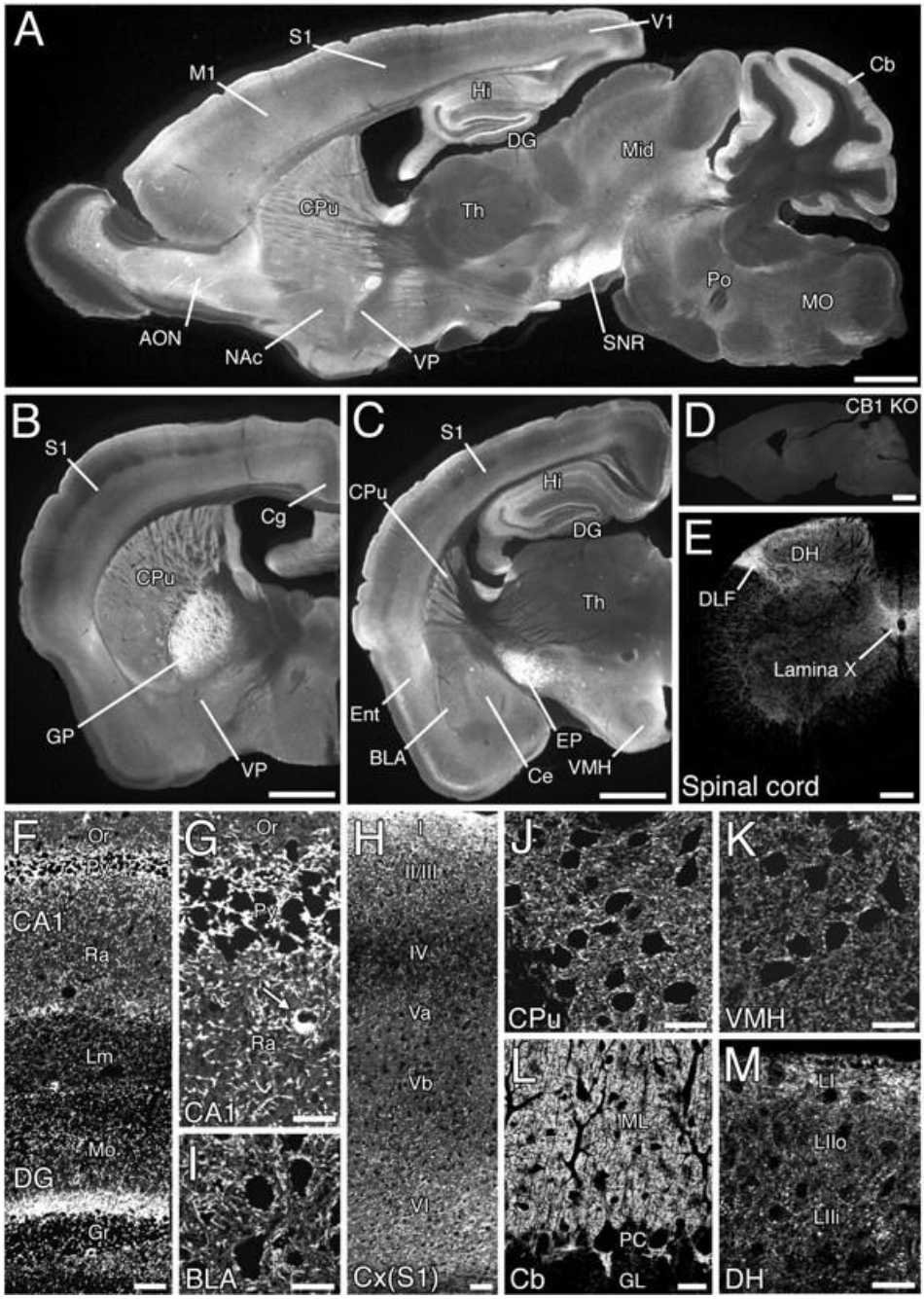


Figure 8: CB1 distribution in mouse brain.

(A) *Immunostaining of CB1 in brain slices of wild-type mice and (**D**) CB1-KO mice. (**B**, **C** and **E**) coronal sections of wild type mice. (**A-C**) High levels of CB1 expression are found in the anterior olfactory nucleus, neocortex, DS, HPC, thalamus, basolateral and central Amy, cerebellum and (**E**) spinal cord. (**D**) CB1 staining is absent in the CB1-KO mouse brain. M1, primary motor cortex; S1, primary somatosensory cortex; V1, primary visual cortex; Cg, cingulate cortex; Ent, entorhinal cortex; DG, dentate gyrus; NAc, nucleus accumbens, GP, globus pallidus; VP, ventral pallidum; Mid,*

midbrain; SNR, substantia nigra pars reticulata; PO, pons; MO, medulla oblongata; EP, entopedoncular nucleus; VMH, ventromedial hypothalamus; DH, dorsal horn; DLF, dorsolateral funiculus. Scale bars: 1 mm (A-C, E), 200µm (D). From (Kano et al., 2009).

CB1 expression is mainly located at neuron pre-synaptic terminals. Upon activation, CB1 modulate glutamatergic, GABAergic, dopaminergic, glycinergic, cholinergic, noradrenergic and serotonergic neurotransmission (Szabo & Schlicker, 2005). CB1 location is located at GABAergic or glutamatergic terminals (Mátyás *et al.*, 2007) but in several structures such as striatum or HPC, CB1 is mainly expressed at GABAergic terminals compare to glutamatergic terminals as illustrated in **Figure 9** (Martín-García *et al.*, 2016).

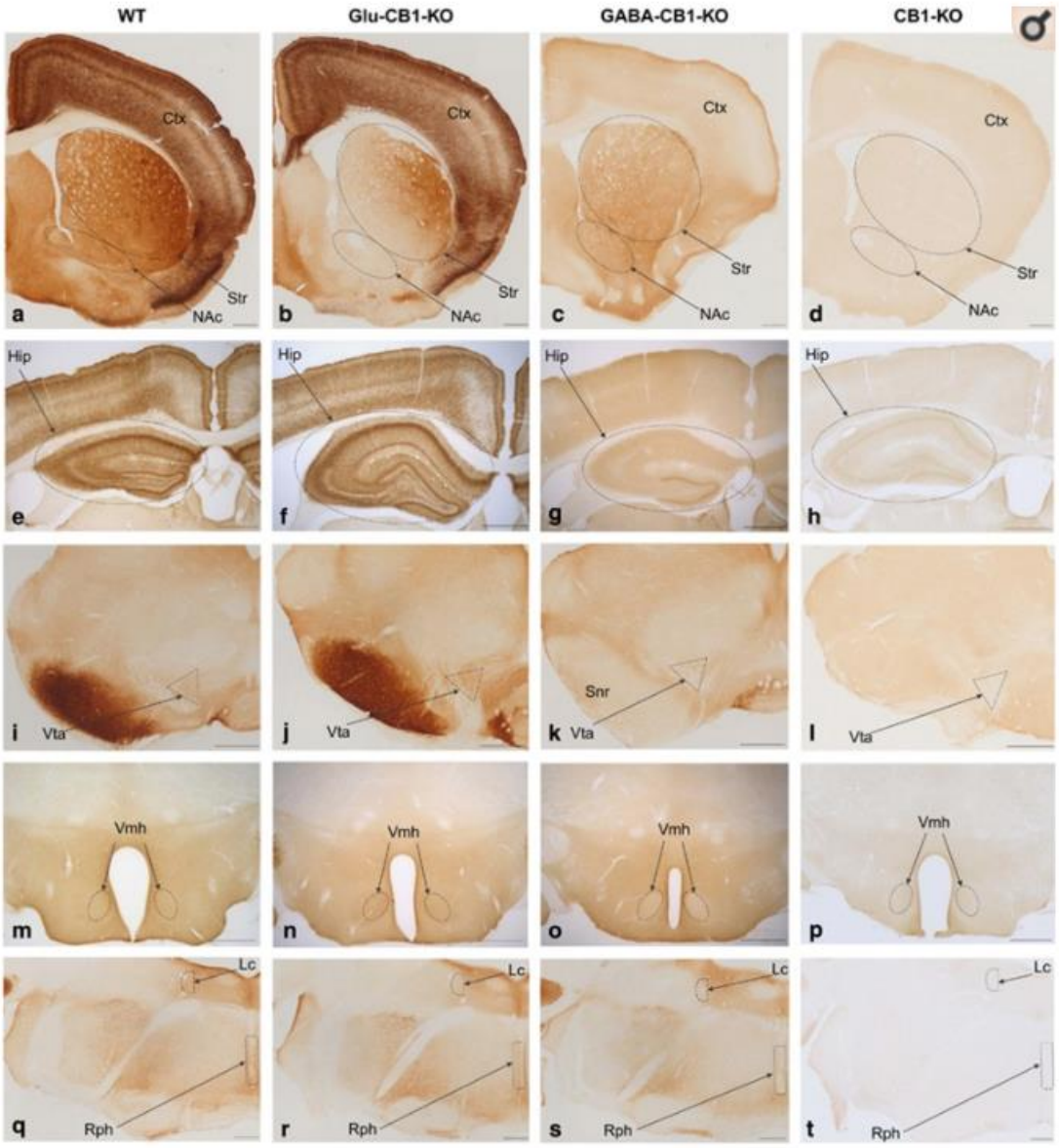


Figure 9: CB1 protein brain expression in WT, Glu-CB1-KO, GABA-CB1-KO and CB1-KO mice. *Cortex (in a–d), striatum (striped oval in a–d), nucleus accumbens (striped oval in a–d), hippocampus (striped oval in e–h), ventral tegmental area (striped triangle in i–l), substantia nigra pars reticulata (in i-l), ventromedial nucleus of the hypothalamus (striped oval in m–p), raphe nuclei (striped rectangle in q–t) and locus coeruleus (striped semi-circle in q–t). Only unspecific diaminobenzidine background is detected in CB1-KO tissue. Relative to WT mice, the Glu-*CB1-*KO showed a mild decrease in CB1 immunoreactivity while the GABA-*CB1-*KO showed a more pronounced decrease. CB1 labeling in dorsomedial and ventral striatum is reduced in Glu-*CB1-*KO (b), whereas substantia nigra pars reticulata lacks CB1 staining in GABA-*CB1-*KO (k). Note the typical strong CB1 pattern in the inner 1/3 of the dentate molecular layer of GABA-*CB1-*KO (g). Ctx, Cortex; Hip, Hippocampus; Lc, locus coeruleus; NAc, nucleus accumbens; Rph, raphe nuclei; Snr, substantia nigra pars reticulata; Str, Striatum; Vmh, ventromedial nucleus of hypothalamus; Vta, ventral tegmental area. Scale bars: 500 µm. From (Martín-García et al., 2016).*

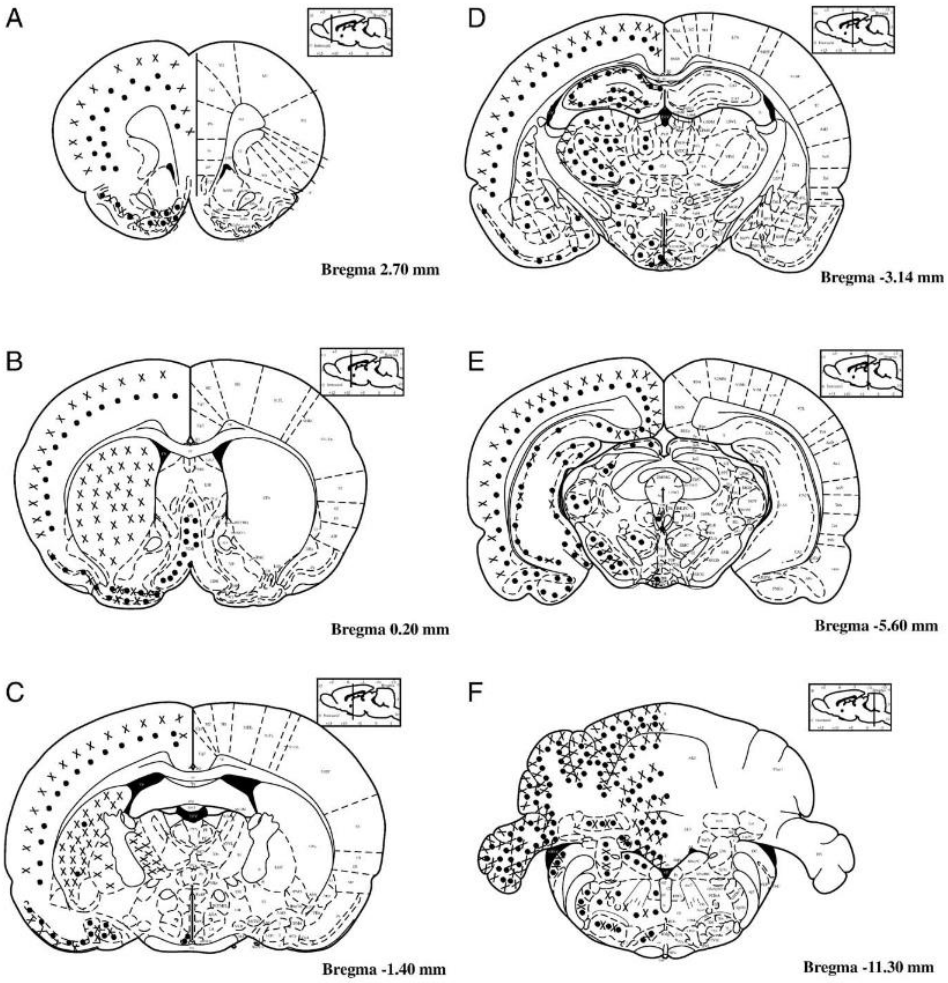
As stated above CB1 is also expressed in astrocytes (Navarrete & Araque, 2008, 2010; Robin *et al.*, 2018). Recent work demonstrated even further the expression of CB1 in astroglial mitochondria in HPC (Gutiérrez-Rodríguez *et al.*, 2018). Interestingly, previous work relying on electron microscopy already showed CB1 expression in hippocampal mitochondria (Bénard *et al.*, 2012). Noteworthy, the same group then demonstrated that hippocampal mitochondrial CB1 regulates memory processes in mice (Hebert-Chatelain *et al.*, 2016).

7. CB2 distribution in brain

Contrary to CB1 large expression in the brain, CB2 expression is far less present in the brain. Early studies even did not detect CB2 mRNA in brain (Galiegue *et al.*, 1995; Schatz *et al.*, 1997; McCoy *et al.*, 1999; Burdyga *et al.*, 2004). Since, several studies suggested CB2 functional expression in brain areas such as brainstem and cerebellum (Van Sickle *et al.*, 2005a; Ashton *et al.*, 2006; Onaivi *et al.*, 2006) as illustrated in **Figure 10A** (Gong *et al.*, 2006). More recently, Schmöle et al. described CB2 expression in microglial cells, notably in HPC, using CB2-GFP BAC transgenic mice **(Figure 10B)** (Schmöle *et al.*, 2015). In several pathological conditions such as Huntington's or Alzheimer's disease, neuroinflammation, neuropathic pain or cancer, CB2 expression was up-regulated in microglial cells (Walter *et al.*, 2003) (for review on these processes see (Atwood & Mackie, 2010; Miller & Devi, 2011). Other studies indicated its expression in neurons as, CB2 activation reduceced prefrontal pyramidal cells firing frequency (den Boon *et al.*, 2012). More recently, it has been demonstrated that CB2 are located on postsynaptic dopaminergic neurons and upon CB2 activation, dopaminergic neuron activity as well as firing was reduced in both mice and rats (Zhang *et al.*, 2014, 2017; Ma *et al.*, 2019). CB2 expression

was also demonstrated in HPC (Brusco *et al.*, 2008b) at a postsynaptic level (Brusco *et al.*, 2008a). Few years later, upon CB2 activation in organotypic cultures of rodent hippocampal slices, the authors observed an increase of glutamate release (Kim & Li, 2015). A year later, the same authors found that deletion of CB2 expression in CA1 neurons enhances spatial memory (Li & Kim, 2017). Very interestingly, modulation of CB2 expression in microglial cells also altered contextual fear memory (Li & Kim, 2017). Back to the HPC, another team found that hyperpolarization of pyramidal neurons in HPC was CB2-dependent independently from CB1 (Stempel *et al.*, 2016). However, the authors noticed a reduction of field excitatory postsynaptic potentials in CA3 elicited by the mixed cannabinoid receptor agonist (11R)-2-Methyl-11-[(morpholin-4-yl)methyl]-3-(naphthalene-1-carbonyl)-9-oxa-1-azatricyclo[6.3.1.$0^{4,12}$]dodeca-2,4(12),5,7-tetraene (WIN55-212), but this effect was not mimicked by [(1R,2R,5R)-2-[2,6-Dimethoxy-4-(2-methyloctan-2-yl)phenyl]-7,7-dimethyl-4-bicyclo[3.1.1]hept-3-enyl]methanol (HU-308), a CB2 specific agonist (see figure 7 in (Stempel *et al.*, 2016)) which dampen the findings from this study. Two studies using DAT-Cnr2-KO, in which CB2 is deleted specifically in dopaminergic neurons, showed alterations anxiety, depressive-like behavior and drugs of abuse reinforcing properties (Liu *et al.*, 2017; Canseco-Alba *et al.*, 2019). Results from the two later studies will be discussed later in part *"**D**: Articles; **I**: cocaine-induced neuroadaptations of the endocannabinoid system in reward-related brain regions: new insights into epigenetic regulations of cannabinoid genes"* regarding CB2 involvement in reward.

A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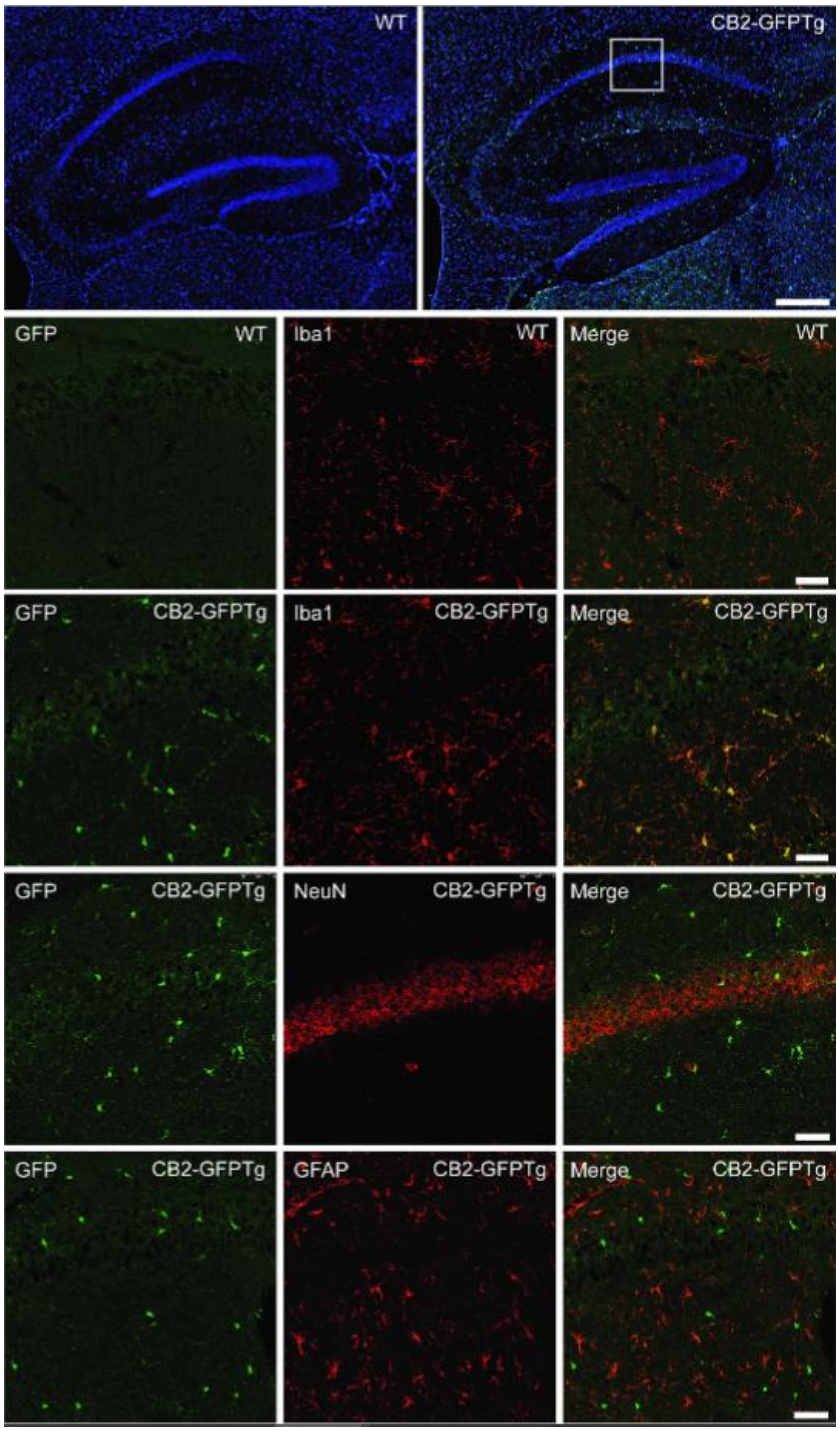

Figure 10: CB2 distribution in mouse brain. [A] *Schematic representation of iCB2-immunopositive structures in the rat brain corresponding to rostro caudal levels (with respect to bregma) +2.70 mm (A), +0.20 mm (B), −1.40 mm (C), −3.14 mm (D), −5.60 mm (E), −11.30 mm (F). Closed circles indicate immunopositive perikarya. X represents immunoreactive fibers and nerve terminals. From (Gong et al., 2006).* ***[B]*** *CB2-GFP expression in the hippocampus of WT and CB2-GFP mice. Immunohistochemical analysis of GFP expression in the brain of CB2-GFPTg mice. CB2-GFPtg mice show GFP expression in the (a, right) which is not present in WT mice (a, left) (scale bar = 250µm). CB2-GFP expression is co-localized with Iba1-staining (b, second row), but not with NeuN (b, third*

row) or GFAP (b, fourth row). First row shows background analysis in WT mice (scale bar = 50µm From (Schmöle et al., 2015)

8. Endocannabinoids and enzymes of synthesis and degradation

The 2 majors' eCBs ligands are the AEA and 2-AG discovered in the early 90's as stated previously (Devane *et al.*, 1992; Mechoulam *et al.*, 1995). Others eCBs have been then described: 2 arachidonoylglycerol ether (Hanus *et al.*, 2001), O-arachidonoylethaloamine (virodhamine) (Porter *et al.*, 2002) and N-arachidonoyldopamine (Huang & Walker, 2006) and lysophosphatidylinositol (a GPR55 ligand) (Oka *et al.*, 2007). Pepcans (also named peptide eCBs) which derived from the α-hemoglobin bind and modulate CB1 activity (Bauer *et al.*, 2012). However, their functionality remains to be further explored, therefore I will focus here only on AEA and 2-AG.

Both eCBs are arachidonic acid derived. They are synthesized on an "on demand" mechanism by cleavage from membrane phospholipids and immediate release through Ca^{2+}-dependent mechanisms in post-synaptic compartment (Ohno-Shosaku & Kano, 2014). Therefore, both act as retrograde messengers to activate both CB1 and CB2. While AEA is a partial agonist at cannabinoid receptors and an endogenous ligand at the transient receptor potential cation channel subfamily V member 1 (TRPV1), 2-AG act as a full agonist at both CB1 and CB2 (Ohno-Shosaku & Kano, 2014; Parsons & Hurd, 2015). Interestingly, AEA also binds intracellularly TRPV1 while 2-AG does not bind TRPV1. This interaction is involved in neuroinflammatory processes (Ross, 2003).

AEA is synthesized by the main enzyme N-arachidonoyl phosphatidylethanolamine – phospholipase D (NAPE-PLD) from NAPE but other pathways are also under consideration (Di Marzo, 2018). AEA is then degraded primarily by the fatty acid amide hydrolase (FAAH) enzyme, which hydrolyses AEA into ethanolamine and arachidonic acid in pre-synaptic neuron (Nicolussi & Gertsch, 2015; Di Marzo, 2018). 2-AG is synthesized from arachidonic acid-containing diacylglycerol (produced by the action of phospholipase C) by a diacylglycerol lipase (alpha (DAGLα) or beta. Again, other pathways of synthesis are considered (Di Marzo, 2018). 2-AG is then mainly metabolized by a monoacylglycerol lipase (MAGL) (about 85%, the others enzymes being α/β-hydrolase domain 6 and 12 (Blankman et al., 2007)) giving glycerol and arachidonic acid in post-synaptic neuron (Nicolussi & Gertsch, 2015; Di Marzo, 2018). I described the main pathways of synthesis and degradation for the two majors' eCBs. The situation is far more complex as authors now mention the "endocannabinoidome" (for recent review see Di Marzo (Di Marzo, 2018))

It has been shown that 2-AG levels are approximately 1000-fold higher than AEA levels throughout brain areas (nmol.g^{-1} versus pmol.g^{-1}) **(Figure 11)** (Buczynski & Parsons, 2010). In **Figure 11**, one can observe that while 2-AG levels are relatively identical throughout brain structures, AEA levels are more variable, with higher levels in HPC and DS compare to other brain areas **(Figure 11)**. In our paper *"**Article 1**: Voluntary cocaine intake modulates the endocannabinoid system in hippocampus"*, we obtained a highly similar profile of eCBs levels (see **supplemental Figure 2**).

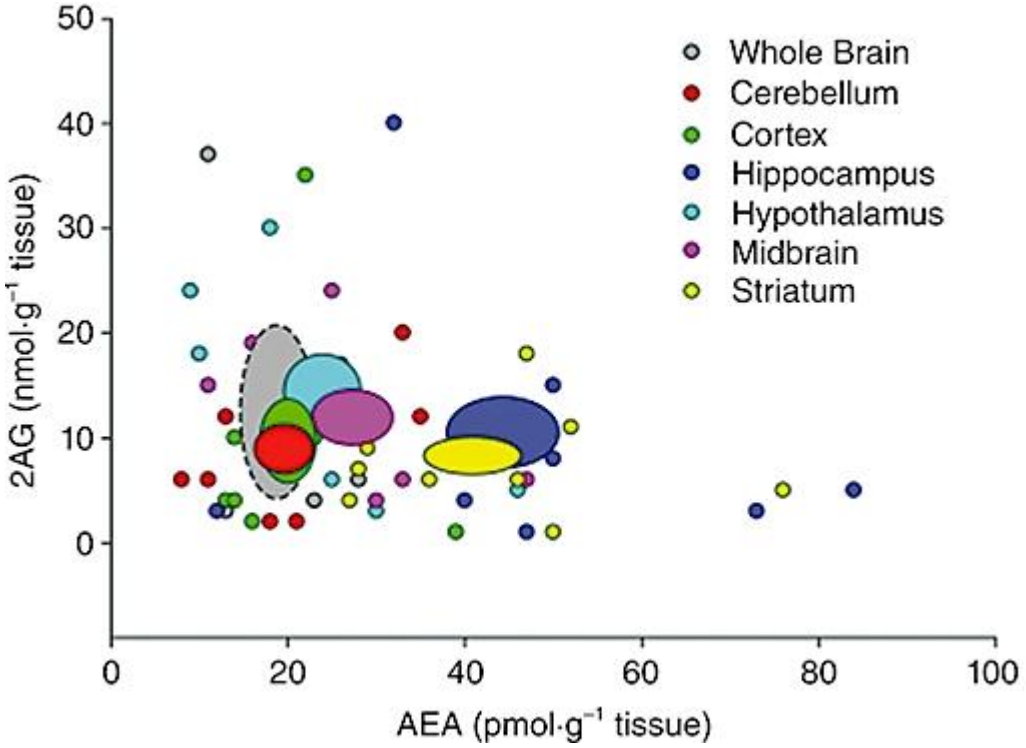

***Figure 11: AEA and 2-AG levels in six brain structures from a meta-analysis**. Each small data point represents the average value of AEA, and 2-AG content reported in an individual study, with average values from at least eight publications presented for each brain region. The mean and standard error of the collective AEA and 2-AG measures are represented as large ovals. From (Buczynski & Parsons, 2010).*

As for other neurotransmitters, the question whether eCBs could be reuptaken in neurons or astrocytes after ligand-receptor interaction quickly raised. This would suppose that eCBs membrane transporter exists. AEA analogs were synthetized and N-arachidonoylphenolamine (AM404) has been proposed as a specific AEA membrane transporter (AMT) blocker (Beltramo *et*

al., 1997). However, AM404 was shown also to inhibit FAAH (Nicolussi & Gertsch, 2015) and activates TRPV1 (Zygmunt *et al.*, 2000), thus questioning the selectivity of this blocker. Since, several putative eCBs membrane transporter (EMT) blocker have been synthetized but the AMT remains to be found at the protein level (for review see Table 1 in Nicolussi et al. (Nicolussi & Gertsch, 2015)). Another model suggests that upon diffusion or facilitated diffusion (AMT) through the membrane, AEA transport would be mediated by intracellular carrier proteins (Maccarrone *et al.*, 2010) to be degraded or to activate TRPV1 intracellularly. Few studies addressed the transport of 2-AG. Briefly, among all the putative EMT blocker synthetized, several inhibited uptakes of both AEA and 2-AG or only 2-AG (see Table 1 in Nicolussi et al. (Nicolussi & Gertsch, 2015)). However, as for AEA, there is still a lack of evidence of EMT at the protein level. As for AEA, an intracellular protein carrier, FABP5, binds 2-AG intracellularly and thus, may mediate its transport in the cytosol. Altogether, eCBS synthesis and catabolism are regulated by specific and different pathways which lead a marked change in terms of eCBs levels in the brain. Thus, these subtle changes of eCBs levels in specific brain structures alter brain activity and physiology.

9. Modulation of synaptic transmission and plasticity

Already in the 1990s, Pitler and Alger noticed that depolarization of post-synaptic neurons mediates suppression of GABA responses in HPC (Pitler & Alger, 1992; Alger *et al.*, 1996). It is only in 2001 that three almost concomitant papers finally demonstrated the involvement of eCBs and CB1 in that process (Kreitzer & Regehr, 2001; Ohno-Shosaku *et al.*, 2001; Wilson & Nicoll, 2001). Since, the involvement of the ECS have been deciphered and two functional categories emerged: eCB-mediated short-term depression (eCB-STD) and eCB-induced long-term depression (eCB-LTD) (Ohno-Shosaku & Kano, 2014). eCB-STD consists in a transient suppression of neurotransmitter release while eCB-LTD is persistent. Interestingly, in DAGLα-KO mice, suppression of synaptic transmission was absent in DS, HPC and cerebellum, highlighting 2-AG as necessary for these phenomenon (Tanimura *et al.*, 2010). Here I will briefly present well characterized forms of eCB-mediated modulation of synaptic transmission, eCB-STD forms:

- Depolarization-induced suppression of inhibition/excitation (DSI/DSE): DSI consists in a transient (few seconds) suppression of inhibitory input onto a neuron following a strong activation (Pitler & Alger, 1992; Lu & Mackie, 2016). DSE is located at excitatory inputs (Kreitzer & Regehr, 2001).
- Metabotropic-induced suppression of inhibition/excitation: same phenomenon as DSI/DSE but occurs upon activation of a $G_{q/11}$ coupled GPCR such as mGluR1, mGlur5, M1,

M3, ORXA, CCK_A and α1 (Kano *et al.*, 2009). Thus, PLC activation promotes the formation of 2-AG which will then diffuse presynaptically to activate CB1.

eCB-LTD consists in a long-lasting inhibition. This process occurs in Amy (Marsicano *et al.*, 2002), in HPC (Chevaleyre & Castillo, 2003), NAc (Robbe *et al.*, 2002) and DS (Gerdeman *et al.*, 2002) to decrease both glutamate or GABA tone. Overall, electrophysiological recordings from these studies described above establish the significance of the ECS in the brain and its role as a synaptic transmission modulator in the CNS.

10. Involvement in pain, memory and learning

The focus of this thesis being the relationships between the ECS and reward, I will only briefly summarize the role of the ECS in pain, memory and learning.

i. Pain

After the discoveries of ECS elements, many studies demonstrated a role for CB1 and eCBs as mediator of nociception and pain (Calignano *et al.*, 1998; Richardson *et al.*, 1998; Walker *et al.*, 1999). Models of acute and chronic pain such as inflammatory or neuropathic pain alter the expression of ECS elements in various peripheral, spinal or brain areas (Starowicz & Finn, 2017). Also, many studies demonstrated how the ECS triggers antinociception and analgesia. As CB1 activation induced psychoactive effects, research is now focusing on other elements associated to the ECS such as CB2, GPR55, TRPV1 and enzymes to try to identify target for treating pain without any secondary effect. A recent review summarizes supraspinal, spinal and peripheral sites of ECS-mediated effect on pain (see (Starowicz & Finn, 2017)).

Regarding clinical trials, a meta-analysis of 28 studies evaluated the effect of cannabinoids (13 with nabiximols, 4 with smoked THC, 5 with nabilone, 3 with THC oromucosal spray, 2 with dronabinol, 1 with vaporized cannabis, 1 with ajuvenic acid capsules and 1 with oral THC) in chronic pain. Conclusions indicated that there was a moderate-quality evidence to support the use of cannabinoids for the treatment of chronic pain and spasticity (Whiting *et al.*, 2015). Interestingly, a study conducted a large survey in 953 patients from 31 countries who used cannabis or cannabinoids to ask about their experiences with the different methods of intake. Herbal non-pharmaceutical products received higher appreciation scores by participants than pharmaceutical products containing cannabinoids (Hazekamp *et al.*, 2013). Many factors should be taken in account to explain this result:

- The legal aspect which depends to the patient country
- The cost as most countries' healthcare expenses do not handle that matter yet

- The medicine availability. For instance, Sativex® (THC-based oromucosal spray) is legal since 2014 in France. However, it is still not available commercially
- Whether the patient is followed-up or not by professionals
- As discussed during the "8th International Conference on Pharmacological Advances and Therapeutic Uses of Cannabinoids" meeting in Strasbourg 2019, it takes time for patients to test different options (THC/CBD ratio, THC/CBD concentration, period of the day, smoked/vaporized/oral intake, etc…). This matter is to date more achievable with cannabis or hemp extracts as many different varieties exist now compared to pharmaceutical products.

ii. Memory and Learning

THC is well known for impairing memory such as short-term memory (Schwartz *et al.*, 1989; Heyser *et al.*, 1993), spatial memory (Lichtman *et al.*, 1995) and learning (Ferrari *et al.*, 1999). CB1-KO mice models confirmed CB1-mediated memory alterations as these mice exhibit an enhanced long-term memory (Reibaud *et al.*, 1999) and an enhancement of both learning in an active avoidance procedure (Martin, Ledent, *et al.*, 2002) and reversal deficits in a Morris water maze task (Varvel & Lichtman, 2002). Interestingly, CB1-KO mice also exhibit LTP enhancement at Schaffer collateral-CA1 synapses (Bohme *et al.*, 2000) which might be the mechanism explaining why CB1-KO mice exhibit learning improvement. Since, a vast amount of animal and clinical studies investigated the relationships between the ECS and memory/learning (for reviews see (Marsicano & Lafenêtre, 2009; Kruk-Slomka *et al.*, 2017)**.** Briefly, findings indicate that CB1 and CB2 bidirectionally modulate memory probably due to many factors such as cannabinoids compounds, doses injected or maybe because of their wide expression in brain regions in various cell types.

Regarding eCBs, the vast majorities of studies investigating learning and memory focused on AEA (Marsicano & Lafenêtre, 2009; Kruk-Slomka *et al.*, 2017) by inhibiting its hydrolysis with cyclohexylcarbamic acid 3-carbamoyl biphenyl-3-yl ester (URB597), a FAAH inhibitor. Up-regulating AEA levels seems to enhance memory. The role of 2-AG remains to be detailed even if recent studies which used the 2-AG hydrolysis inhibitor, 4-nitrophenyl 4-(dibenzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (JZL184), a MAGL inhibitor, indicate memory facilitation in rodents (Busquets-Garcia *et al.*, 2011; Steinmetz & Freeman, 2016; Ratano *et al.*, 2018).

11. Relationships between the endocannabinoid system, cocaine and palatable food

These aspects are detailed in the part describing the articles 1 and 3. I describe further the impact of cocaine on the ECS in section *"**Article 1**: Voluntary cocaine intake modulates the endocannabinoid system in hippocampus"* as an introduction to our paper. In that same section, I also describe how the ECS affect cocaine sensitization, cocaine-CPP and voluntary cocaine intake. Similarly, I represent further the effect of palatable food intake on the ECS in section *"**Article 3**: Binge-sucrose induces endocannabinoid system alterations and transcriptomic adaptations in the NAc"*. I also define the impact of the ECS on palatable food intake.

Thesis statement

B. Thesis statement

At the beginning of my PhD, I was interested in unraveling cocaine-induced modifications of the ECS to better outline its role in cocaine addiction, which is still unclear. Indeed, CB1 antagonist, such as the Rimonabant, decreased cocaine preference and cocaine seeking in rats. Regarding CB2, agonist such as JWH133 decreased cocaine preference but increased breakpoint for cocaine-SA in rats. Such discrepancies made us want to better grasp these modulations in rats. Previous studies investigated these regulations by testing the effects of cocaine sensitization on the ECS. We decided to use a cocaine-SA paradigm to evaluate the impact of voluntary cocaine intake on the ECS. Therefore, our objective through our work was to study the modifications of the ECS induced by cocaine to decipher the ECS involvement in cocaine addiction in specific brain reward-related regions. To do so, we used a screening approach at the molecular scale in brain regions. We quickly focused the study on CB1 expression and functionality due to the regulations we observed in the HPC. Then, we used innovative procedures in the field of neuroscience to investigate epigenetic processes by analyzing histone modifications and chromatin changes at endocannabinoid genes.

In the middle of my PhD, I went to Canada to perform a collaborative work with M.C Olmstead team in Kingston, Ontario, Canada. We were interested in binge-sucrose induced adaptations on the ECS as the latter also modulate food reward. Indeed, Rimonabant decreased food intake while agonist, such as the WIN 55,212-2, promoted it in rats. At the beginning of this project two questions quickly arised: is there an addiction to sugar? If so, does it induce the same type of modification, at the ECS level, compare to cocaine addiction? Obviously, we first performed the same work as described previously, meaning a screening of the binge-sucrose induced modifications at the ECS genes and tone in brain reward-related regions. Then we conducted a transcriptomic approach on a wider scale (RNA-Seq) to examine whether sucrose could induce similar or different pathways in the NAc compare to cocaine. Overall, our goal was not only to better understand the involvement of the ECS in reward but also to identify a target, potentially at the epigenetic level, by acting on the ECS to treat cocaine addiction and, potential sugar-use disorder.

Finally, I participated to three side projects during my PhD. First, in parallel to the cocaine-ECS project, we were interested in cocaine-induced modifications of the OS also playing a crucial ole in reward. Secondly, our team was testing the effect of an mGluR4/7 agonist on cocaine intake and seeking. I participated to this project by performing the molecular and protein analysis. Thirdly, our team had some data showing the role of CB2 in the activation of epigenetic factors such as Mecp2 and HDAC2. I participated to additional molecular experiments in this study.

Protocol optimization

C. Protocol optimization

During my thesis, I used several technical approaches, from behavior to molecular aspects. Here, I will detail some of them, specifically when optimization steps were necessary to overcome some difficulties. The other methodological approaches are detailed in each method section of the different articles.

I. RNA extraction for RNA sequencing: troubleshooting with sample variability

i. Sample preparation

We classically used the Trizol method to extract RNA from brain tissues (Befort *et al.*, 2008). Using the Trizol method, we obtained large mRNA amounts but with strong variability. For NAc samples (n=29), the amount of RNA was 7,90 ± 0.9 ng RNA/sample. The mean weight of NAc sample was 17 mg. We observed some variability in this preparation **(Figure 12)**, which could arise from several factors: the RNA preparation itself, the size of the sample, the tissue or its microdissection. However, this is most likely due to the RNA preparation or the tissue as we only encountered such variability with the HPC and no other structures (data not shown). Also, the 260/280 ratios were quite low, suggesting a lower quality of the RNA extraction **(Figure 12)**. Nevertheless, this was not a problem for further use in qPCR experiment as we were always starting from the same amount of RNA to perform the experiment (750 ng).

For RNA sequencing, we decided to better standardize this preparation by using specific column for small sample preparation (kit from Macherey Nagel, (740955.50, France) **(Figure 12)**. Total extracted RNA from NAc samples (n=20) was reduced compared to Trizol method, and we obtained less variability **(Figure 12)** as well as a better 260/280 ratio **(Figure 12)**.

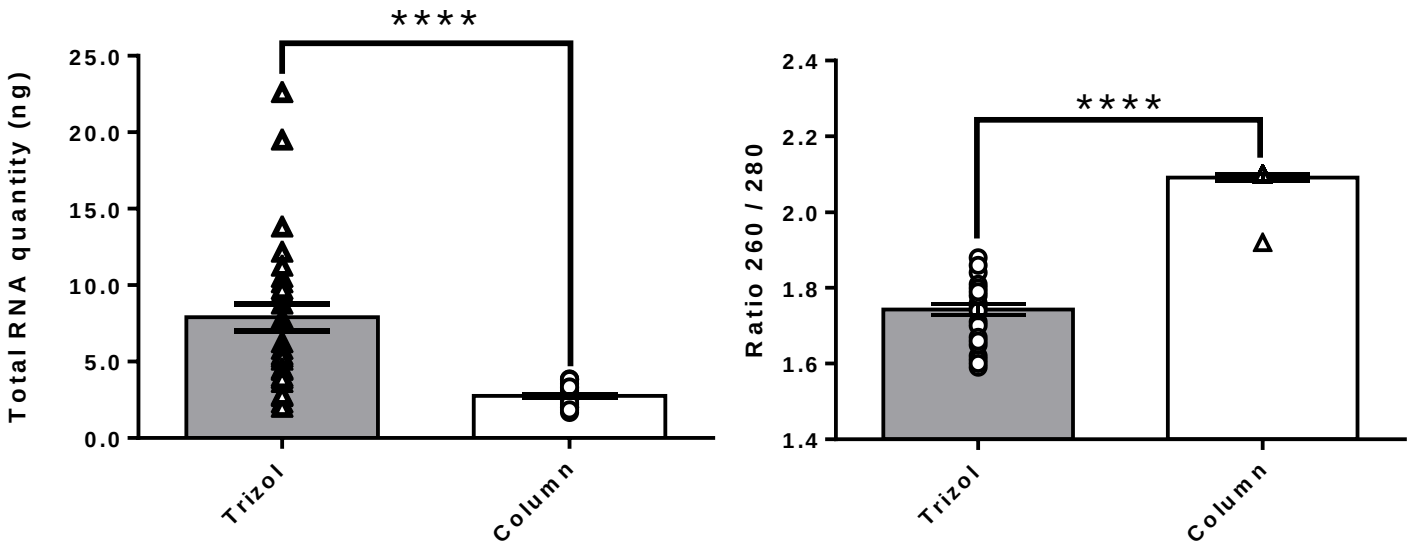

Figure 12: Total RNA and 260/280 ratios following RNA extraction. Data represented show the total RNA extracted and its quality obtained with either Trizol or the Macherey-Nagel Kit (Column) extraction method, with NAc samples.

ii. Quality control for RNA seq

After extraction with the Macherey-Nagel kit, 20 samples of RNA were sent to the GenomEast platform (http://genomeast.igbmc.fr/) at IGBMC. First, the RNAs amount have been measured and then validated (quality control test, see **Figure 13** below). The integrity of RNA was evaluated using the 28S to 18S ribosomal RNA ratio. Briefly, to obtain a perfect RNA integrity (=10), with the 28S band supposed to be twice the size of the 18S band. The RNA integrity per sample is represented below.

Sample ID	Concentration (ng/μl)	Volume (μl)	Quantity (ng)	Integrity	Validated
KTBT1	170.00	13.00	2210.00	OK	Yes
KTBT2	140.00	13.00	1820.00	OK	Yes
KTBT3	73.00	13.00	949.00	OK	Yes
KTBT4	90.00	13.00	1170.00	OK	Yes
KTBT5	111.00	13.00	1443.00	OK	Yes
KTBT6	171.00	13.00	2223.00	OK	Yes
KTBT7	87.00	13.00	1131.00	OK	Yes
KTBT8	88.00	26.00	2288.00	OK	Yes
KTBT9	80.00	10.00	800.00	OK	Yes
KTBT10	153.00	10.00	1530.00	OK	Yes
KTBT11	128.00	15.00	1920.00	OK	Yes
KTBT12	140.00	13.00	1820.00	OK	Yes
KTBT13	92.00	20.00	1840.00	OK	Yes
KTBT14	110.00	15.00	1650.00	OK	Yes
KTBT15	105.00	13.00	1365.00	OK	Yes
KTBT16	55.00	13.00	715.00	OK	Yes
KTBT17	81.00	13.00	1053.00	OK	Yes
KTBT18	156.00	13.00	2028.00	OK	Yes
KTBT19	94.00	13.00	1222.00	OK	Yes
KTBT20	87.00	13.00	1131.00	OK	Yes

RNA integrity number per samples

KTBT1: RIN:9.90
KTBT2: RIN:9.70
KTBT3: RIN:9.40
KTBT4: RIN:9.50
KTBT5: RIN:9.40
KTBT6: RIN:9.50
KTBT7: RIN:9.50
KTBT8: RIN:9.80
KTBT9: RIN:9.50
KTBT10: RIN:9.70
KTBT11: RIN:9.70
KTBT12: RIN:9.60
KTBT13: RIN:9.70
KTBT14: RIN:9.70
KTBT15: RIN:9.60
KTBT16: RIN:9.60
KTBT17: RIN:9.70
KTBT18: RIN:9.40
KTBT19: RIN:9.30
KTBT20: RIN:9.10

Figure 13: Sample validation report. *Following the validation of RNA integrity, quantification has been performed by Qubit.*

iii. Conclusion

Using the Macherey-Nagel kit to overcome sample variability, we obtained 20 consistent samples of not only good quality but also excellent integrity. Thus, these samples were further processed for performing RNA sequencing.

II. Western blotting experiments: targeting cannabinoid receptors CB1 and CB2 and cannabinoid interacting protein.

To evaluate cocaine-induced neuroadaptations at the ECS in reward-related brain regions, we wanted to assess the protein levels of both CB1 and CB2. For sample preparation for western blot analysis, we performed a classical method, consisting of the following steps. Frozen samples were dounced homogenized in sucrose 0.25M and we collected a first fraction entitled "Homogenate" condition. Then, the rest of the samples was centrifuged at 1100g, 10min at 4°C. We collected the second fraction corresponding to the "Membrane enriched" condition. For the final step, samples from the same previous preparation were ultra-centrifuged at 100000g, 30 min at 4°C giving the last condition "Membranes" (classically used in GPCR analysis (Sim *et al.*, 1995). Samples were aliquoted and then processed for WB analysis with distinct antibodies. Detail protocol is written in our article *"Voluntary cocaine intake modulates the endocannabinoid system in hippocampus"* in section *"**D**: Articles; **I**: cocaine-induced neuroadaptations of the*

endocannabinoid system in reward-related brain regions: new insights into epigenetic regulations of cannabinoid genes". Briefly, identical amount of proteins 20 µg of homogenate and 30µg of membrane preparations were separated on a stain free polyacrylamide gel 4-15% (Biorad, France) and proteins were transferred onto a PVDF membrane (Biorad, France). Resulting blots were then blocked in PBS-I-block (Tropix, Applied Biosystems), 0.1% Tween 20 buffer for 1h. An overnight incubation at 4°C with CB1 antibody was processed (primary rabbit anti-CB1 antibody). Blots were washed and then incubated in biotinylated secondary goat anti-rabbit anti-body (1:50 000) for 1h at room temperature (Jackson ImmunoResearch Laboratories, Inc). Antibody binding was revealed by chemiluminescence (ECL Prime, GE healthcare, Piscataway, NJ, USA) detected using the ChemiDoc Imager (Biorad, France). When needed, normalization was performed using stain free (total protein) as in (Gürtler *et al.*, 2013)

i. Targeting CB1

Antibodies for GPCR often present a low specificity and when starting our experiments, we performed an overview of the literature to try to find the best candidates. First tests were performed on enriched membranes and membranes samples with three CB1R rabbit antibodies: Abcam 1:300 (ab23703); Frontiers 1:300 (AF380); Santa-Cruz (SC-10066)1:300. The first assays with the three antibodies revealed a quite heterogeneous CB1 staining **(Figure 14)**. The Abcam antibody revealed a band corresponding to CB1 expected size (≈ 52 kDa), with many other bands
.

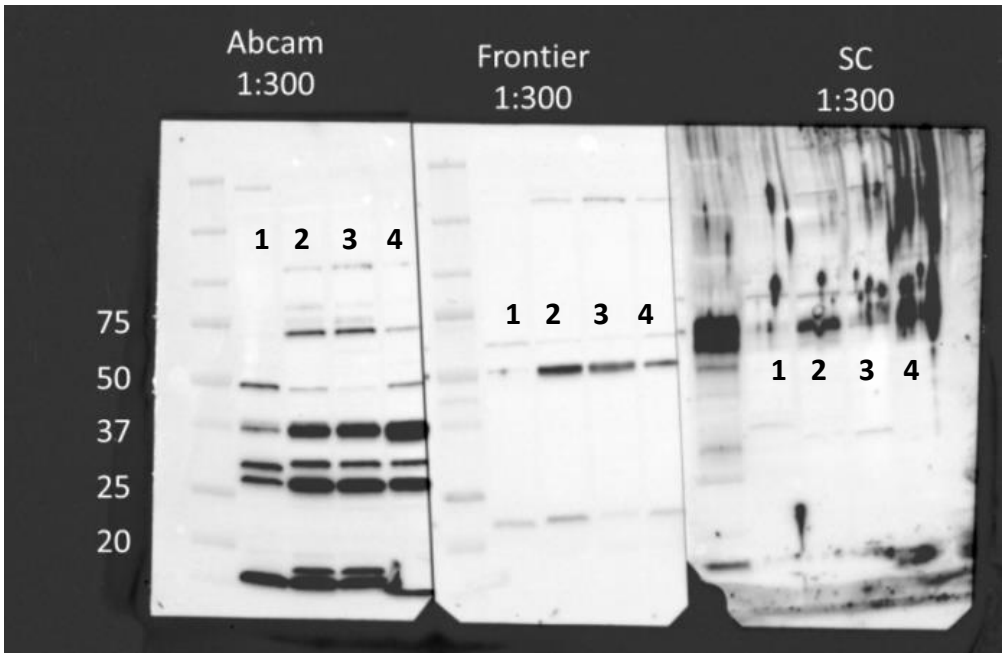

Figure 14: CB1 staining on WB using three different antibodies (Abcam; Frontiers; Santa-Cruz). *CB1 expected size is ≈ 52 kDa. Two different preparations were used for each antibody, "Membranes" from mice striatum (Well 1) and "Enriched membranes" from rat DS, HPC and not defined (Wells 2-3-4 respectively). Molecular weights are on the left lane of each blot. Gel 12%.*

Unfortunately, too many bands with strong intensities occurred by using this antibody making the analysis difficult. The Frontiers antibody unveiled a strong band at the expected size despite two other weak bands. Finally, the Santa-Cruz antibody showed a very heterogenous and poor staining making the use of this antibody inadequate. We decided that using the Frontiers antibody would be the best option to detect CB1 expression with confidence, in our conditions. We then proceeded to test the validity of the Frontiers antibody on DS samples of rats prepared in different conditions (Homogenate, membrane enriched and membranes (see above). As CB1 is a membrane receptor, we thought that a better detection could occur in such samples. The corresponding staining is represented in **(Figure 15)**.

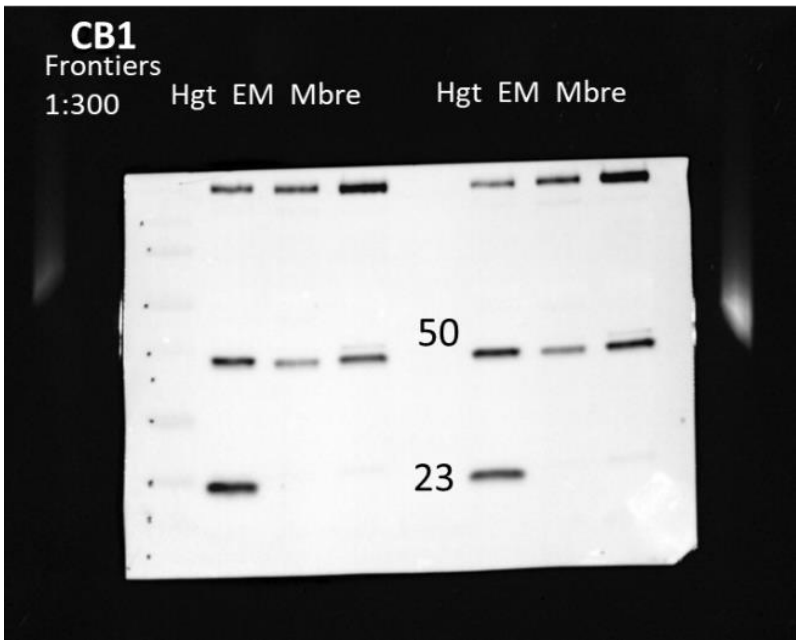

Figure 15: CB1 staining on WB using the Frontiers CB1 antibody. *CB1 expected size is ≈ 52 kDa. Three different preparations were evaluated: "Homogenate (Hgt)", "Enriched membranes (EM)" and "Membranes (Mbre)" from rat DS. Gel 12%.*

The profile was rather clear, with a nice detection of the band at the expected size for CB1. We decided to work with the homogenate conditions as the staining was correct and working with this condition was easier to prepare. Therefore, experiments were performed in this latter condition, on samples from rats self-administering cocaine or their saline controls. Our preliminary results indicated no significant changes in either PFC, DS or HPC. In the meantime, we acquired mouse CB1-KO brain samples (Dr Nozaki, Zimmer's lab, Bonn University). We prepared homogenate and membrane samples from total brain in the same conditions as for our rat experiments. Using the Frontiers antibody, we unfortunately detected a band in CB1-KO samples at the expected size (52kDa), in both types of preparation **(Figure 16)**. This result using one of the best control possible to verify antibody specificity was very disappointing.

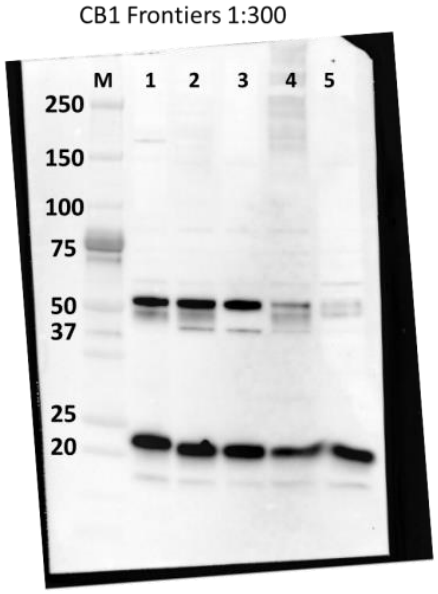

Figure 16: Frontiers antibody test using CB1-KO samples on WB. *Five different conditions were tested: homogenate from rat total brain (Well 1), homogenate from WT mouse total brain (Well 2), homogenate from CB1-KO mouse total brain (Well 3), membranes from WT mouse total brain (Well 4), membranes from CB1-KO mouse total brain (Well 5). "M" represents the weight marker. Gel 12%*

In the meantime, we ordered another antibody targeting CB1 produced by Cayman **(**10006590**)** and tested it in similar conditions. Results were more encouraging **(Figure 17)**. The global staining

profile was different than the Frontiers Ab, but we found no CB1 staining in CB1-KO mice using the antibody from Cayman (1:500), at the expected size, while a clear band was present in WT controls or CB2-KO samples in both types of preparation.

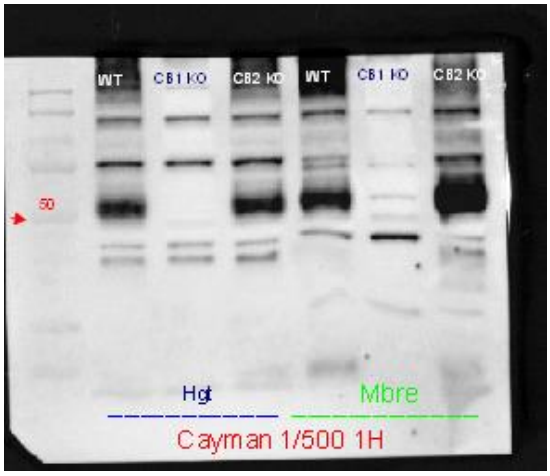

Figure 17: Cayman antibody test using WT, CB1-KO and CB2-KO samples on WB. *Homogenates (Hgt) and membranes (Mbre) preparations were tested for the three different conditions. Gel 12%.*

In conclusion, we lost quite a lot of time and tissues using a "non-specific" antibody, at least in WB analysis. Using the antibody from Cayman, we finally studied the regulation of CB1 protein following cocaine-SA and our results are represented in our article *"Voluntary cocaine intake modulates the endocannabinoid system in hippocampus"*.

ii. Targeting CB2

Targeting CB2 with precision is very challenging and many studies struggled to find specific antibodies (Marchalant *et al.*, 2014). The expected size of CB2 is 48kDa. After discussing with Zimmer's lab, they advised to use an antibody from Abcam (ab3561). We performed similar experience to set up our experimental conditions. Our results indicated that this antibody was non-specific as we detected a strong staining in CB2-KO samples at the expected size **(Figure 18)**.

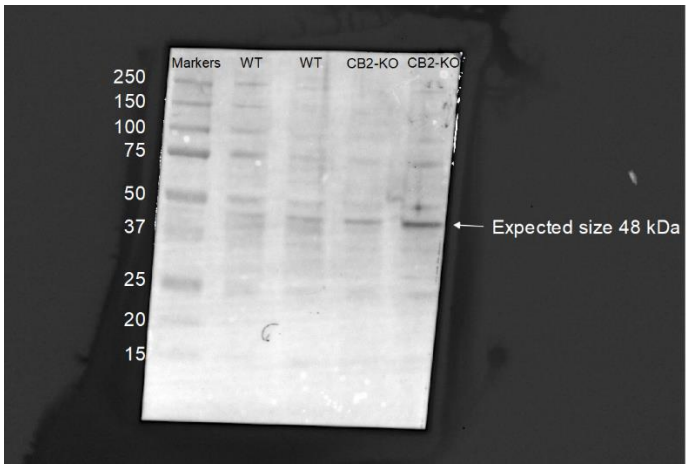

Figure 18: CB2 antibody test using WT and CB2-KO samples on WB. *Homogenates and membranes preparations were tested for the three different conditions with CB2-Ab from Abcam. Gel 12%.*

We decided to stop our investigation there and did not take the time to try other antibodies targeting CB2.

iii. Targeting CRIP1A

As stated in section *"**A**: Introduction; **IV**: the endocannabinoid system"* of the introduction, cannabinoid interacting proteins such as CRIP1A or SGIP1 are regulating CB1 functionality. Thus, analyzing their expression could help better understanding the cocaine-induced functional adaptations on the ECS. When we started to investigate that matter, we did not find any SGIP1 antibodies available. Thus, we focused on CRIP1A (expected size around 18 kDa). Using an antibody from Abcam (ab167087), our results showed 2 bands around the expected size **(Figure 19)** (1 small band around 20 kDa and 1 bigger band around 18 kDa). Even if the bigger band is closer to the expected size, we were not confident about using this antibody as the two bands were rather close. Moreover, as CRIP1A is supposed to be anchored in the membrane its expression should be also detected in "enriched membrane" preparation. However, we detected only the small band around 18 kDa in the "enriched membrane" preparation **(Figure 19)**. Therefore, we did not pursue the investigation of CRIP1A expression as we already experienced problems with western blot experiments.

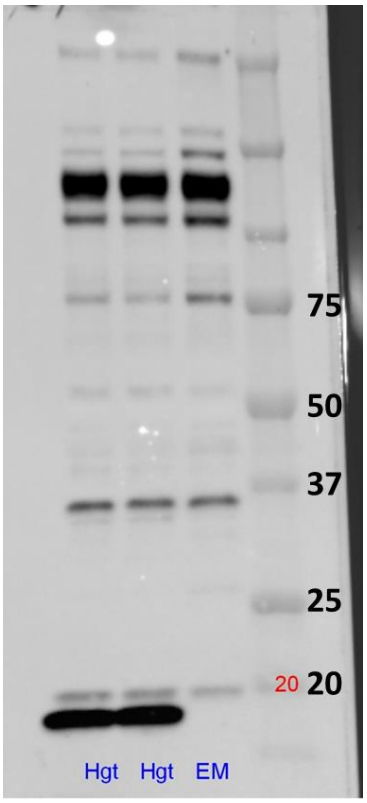

Figure 19: CRIP1A antibody tests on rat total brain samples by WB. *Hgt: homogenate preparation; EM: enriched membrane preparation. Gel 4-15%.*

III. Mass spectrometry: troubleshooting for detecting and measuring Anandamide in brain samples

To measure endocannabinoids levels, the only reliable method is the mass spectrometry. To do so, we collaborated with Yannick Goumon (INCI, Strasbourg). With the help of Virginie Chavant, we tested various extraction methods described in the literature (Buczynski & Parsons, 2010). Using these methods, I briefly describe the results we obtained here on HPC and DS test samples:

- Methanol: 2AG isomerization into 1AG

- Ethanol: 2AG isomerization into 1AG

- Methanol/chloroform: 2AG isomerization into 1AG

- Toluene: poor detection of 2AG and AEA

- Ethyl acetate: poor detection of 2AG and AEA

- Acetonitrile: good detection of 2AG and AEA

We decided to perform the eCBs measures using an acetonitrile extraction, as it appeared to be the best solution to measure precise amounts of eCBs. To do so, brain samples were first sonicated in H_2O, samples were then taken and mixed in acetonitrile 100% containing known fixed amounts of deuterated internal standards: 400.26 pmol of D8-2AG (sc-480539; Santa Cruz, Heidelberg, Germany) and 100.15 pmol of D4-AEA (Tocris/Biotechne, Lille, France) (for detailed methods, see our article "*Voluntary cocaine intake modulates the endocannabinoid system in hippocampus"* or see **Figure 20** for an overview of our protocol). Then, samples were loaded onto an Accucore C18 LC column (column 1, 1x100 mm, 2.6 µm) heated at 40°C. Qualification and quantification were performed in MRM mode. All amounts of endocannabinoids measured in samples fit within the standard curve limits, with typical analytical ranges (the range of amounts that can be accurately quantified) from 1 fmol – 100 pmol to 150 fmol – 100 pmol. Precision (CV% between repeated injections of the same sample) values were <1% for same-day measurements and <5% for inter-day measurements. The amount of 2AG (nmol) and AEA (pmol) observed were normalized according to protein concentrations (mg) as this normalization method is more precise compare to a normalization according to the weight of the original tissue.

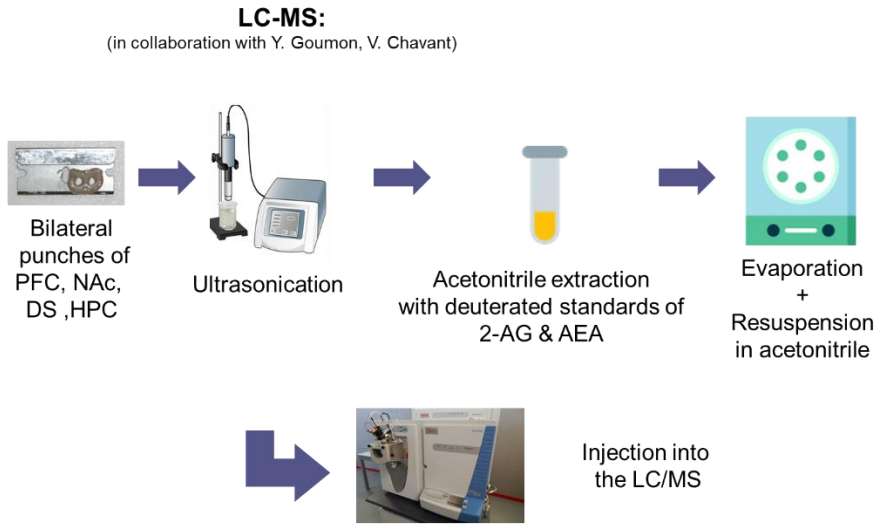

Figure 20: Overview of the extraction and measure of endocannabinoids. *Punches of reward-related regions were ultrasonicated in water. Then, eCBs were extracted using an acetonitrile extraction method by adding deuterated standards in the mix. Following evaporation of the*

extraction, samples were injected in the LC/MS for measurements. Extraction and injection of samples were the two steps we optimized in our analysis (see below).

We conducted the first assays using only HPC and DS tissues. We had a bigger amount of tissue to start with (25-30mg) compared with NAc or PFC samples (15-20mg) and also, these structures are known to contain higher endocannabinoid levels according to the literature (AEA: 45 pmol.g^{-1} and 2AG: 10 nmol.g^{-1}, see chapter "The endocannabinoid system" (Buczynski & Parsons, 2010)). Unfortunately, we were not able to measure precise amounts of AEA levels in both tissues, even by pooling samples from three animals together **(Figure 21)**. We had the chance to discuss this issue with Dr Finn D.P. (Gobira *et al.*, 2019), who advised us to switch the column 1 to a microbore C18 ODS column (column 2, 1x100mm, 3µm). This really improved our conditions and we were finally able to detect and measure AEA levels above the minimal detection value (1000) as pictured in **(Figure 21)**.

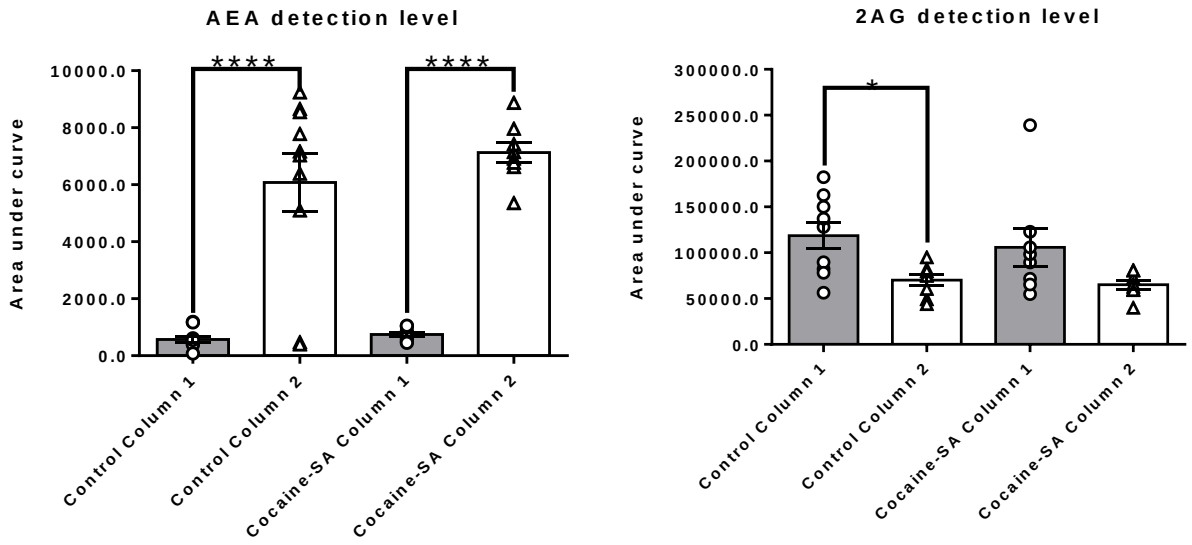

Figure 21: AEA and 2AG detection level in DS with column 1 and 2. *Histograms represent the area under the peaks of eCBs detection. n = 8-9 per groups; * = p<0.05; **** = p<0.0001.*

The detection of 2AG decreased in the control group following the change of the column but the detection was less variable, and it did not affect the precision of the measure as the detection levels were still excellent. In conclusions, after almost a year of optimization, we achieved a good detection of AEA in all structures under investigation (PFC, NAc, DS and HPC) without pooling samples from different animals. We are now thinking about extending these measures to other

brain structures such as the HYP, Hb, VTA and RMTg. However, AEA levels in NAc being low, these raised concerns about whether we will obtain a good detection of AEA levels in these structures that are smaller (<15mg) than NAc. Unfortunately, we did not have the time or the resources to perform these tests during my thesis, but the team will investigate that matter giving the gene expression changes we observed in these structures.

IV. RNA-Seq analysis: troubleshooting for the analysis of differentially expressed genes in cocaine animals compared to controls

Many genome-wide studies investigated the transcriptomic adaptations following cocaine use. However, only few of them used a cocaine-SA paradigm, as reviewed in De Sa Nogueira et al. (De Sa Nogueira *et al.*, 2019). We conducted an RNA-Seq approach with NAc samples of rats who underwent cocaine-SA and binge-sucrose in order to compare cocaine versus sucrose adaptations at the transcriptomic level. Also, to our knowledge, very few studies investigated the effects of palatable diet ,and no study analyzed the consequences of sugar intake, on brain transcriptome. The RNA-Seq data concerning the binge-sucrose analysis is discussed in our report *"Transcriptomic analysis of binge sucrose-induced neuroadaptations: a focus on the endocannabinoid system"*. Surprisingly, we faced difficulties concerning the analysis of cocaine data as no differentially expressed genes could be highlighted in NAc, using standard way of analysis (see attached document **Annex 2** and our article *"Transcriptomic analysis of binge sucrose-induced neuroadaptations: a focus on the endocannabinoid system"*). This result was quite surprising as previous studies using transcriptomic approaches indicated hundreds of cocaine-regulated transcripts in NAc regardless of the protocol (De Sa Nogueira *et al.*, 2019). This result is explained by a strong variability/dispersion of the samples (see PCA analysis in **Annex 2**). We decided to look more deeply into our raw data and performed another analysis. Using the online database OMIM (https://www.omim.org), we found 94 entries/genes related to the term "cocaine addiction" represented in **TABLE 5** below. I added the p-value from our analysis as adjusted p-values in our study were above 0.99. Obviously, using only p-values is highly unadvisable as it does not account for multiple testing problem and false discovery rate. Using unadjusted p-values would only lead to the discovery of meaningless biology but our goal is only to verify whether our result can fit with the literature.

Gene	Log2 FC (Cocaine vs saline)	P-value (Cocaine vs saline)		Gene	Log2 FC (Cocaine vs saline)	P-value (Cocaine vs saline)
Abcb1a	-0,024819106	0,789359226		**Grk6**	-0,030358225	0,614271974
Adgrl3	0,00951957	0,919167754		**Grm2**	0,202698989	0,040844666
Adh1				**H3c4**	-0,011626979	0,529189191
Adrb2	-0,099480064	0,312566221		**Hcrt**	-2,95E-05	0,997893808
Adrb3	0,007611068	0,771885271		**Homer1**	0,050922531	0,608674864
Ago2	0,029863179	0,75543939		**Homer2**	0,053763068	0,58893239
Agrp	-0,170988171	0,017181568		**Htr1b**	0,158975409	0,106341155
Aldh1a1	-0,122258848	0,218970655		**Htr2a**	-0,027327506	0,772342857
Aldh2	-0,074905034	0,258270764		**Htr2b**	-0,012920128	0,39844828
Ankk1	-0,004517014	0,772179855		**Htt**	0,020920536	0,81602805
Bdnf	0,176663069	0,012202263		**Irf4**	-0,088489447	0,369440404
Cartpt	0,106851136	0,272907087		**Kcnj9**	0,152733676	0,102099356
Cckar	0,039721853	0,410787569		**Lig4**	0,035192923	0,652717852
Ccnd1	-0,072452379	0,457232394		**Lmo1**	0,037452714	0,695156243
Ccnl1				**Mapk3**	-0,091691167	0,290179649
Cdk5	-0,012665178	0,852925915		**Mef2a**	-0,010561234	0,913422754
Ces2h	0,031747049	0,460521865		**Mef2d**	0,003014535	0,975460151
Chrna3	-0,056254428	0,568864753		**Mitf**	0,060455485	0,532521369
Chrna4	0,074198074	0,445391192		**Nacc1**	-0,020555967	0,741545484
Chrna5	-0,012819412	0,884047967		**Nr0b2**	0,020971848	0,425454649
Chrnb2	0,1292105	0,181741037		**Nr1i3**	-0,01276925	0,62412427
Clock	0,088416979	0,286470356		**Nr3c1**		
Creb1	0,146113401	0,105574912		**Ntrk2**	0,01630625	0,834370184
Cyfip2	-0,059988812	0,495433266		**Nudt1**	-0,123194938	0,206713668
Cyp2a4				**Oprd1**	0,06722786	0,452845864
Cyp2a5				**Oprk1**	0,185732651	0,0615253
Dbh	0,021134573	0,432528182		**Oprm1**	0,093063732	0,220567193
Dlg4	-0,110994363	0,155878852		**Pink1**	-0,032431384	0,68230947
Drd2	-0,044176941	0,635334047		**Plat**	0,082646219	0,369550292
Drd3	0,072818803	0,392682008		**Pomc**	-0,175647578	0,066135261
Drd4	0,011360597	0,531075456		**Pparg**	0,129774974	0,188022761
Ehmt2	-0,123653707	0,019241515		**Prkcz**	-0,036800249	0,620709712
Enpp1	0,168015116	0,090960865		**Prlhr**	0,131702082	0,137810274
Faah				**Raf1**	-0,009502724	0,870842447
Fkbp5	-0,157711683	0,109151935		**Rarb**	0,045714831	0,625154089
Fosb	-0,021196923	0,830873678		**Rxrb**	-0,151657439	0,026872433
Gabbr2	0,116732744	0,185061136		**Rxrg**	0,075062611	0,450238368
Gabra1	0,217785586	0,022592662		**Sdc3**	0,077307559	0,324943463
Gabra2	0,123915337	0,188264568		**Slc18a1**	-0,17241671	0,083147706
Gdnf	0,019562368	0,781146541		**Slc18a2**	0,013792907	0,756339822
Ghrl	-0,004591935	0,681154652		**Slc6a2**	0,021356338	0,384863978
Gpr1	6,88E-06	0,999770867		**Slc6a3**	0,031230226	0,416701706
Gpr3	0,143555034	0,132445194		**Slc6a4**	-3,17E-05	0,997815475
Grasp	-0,137149209	0,164804051		**Spred1**	0,086129506	0,334823286
Gria1	0,146373667	0,108817838		**Tacr1**	0,198283992	0,04592957
Gria2	0,002360761	0,979347457		**Tph2**	-0,07602916	0,414948819
Gria3	-0,026718341	0,768026247		**Ucp3**	0,008034814	0,89631894

Table 5: genes related to "cocaine addiction" according to OMIM website. *Their respective expression and associated p-value from our analysis is represented. Black boxes indicate that this gene was not found in our analysis.*

If we focused on the 11 genes whose p-values are under 0.1 we obtained interesting genes related to cocaine such as:

- ***Bdnf*** (brain derived neurotrophic factor): its role in cocaine intake has been well described as BDNF injections directly into NAc promoted cocaine intake while BDNF inhibition prevented it in rats (Schoenbaum et al., 2007). More recently, BDNF injections into NAc inhibited cocaine seeking in rats (Bobadilla et al., 2019).

- ***Agrp*** (Agouti Related Peptide): using an analog of AgRP (the AgRP 83-132, an inverse agonist of MC4r) directly injected into NAc, a study found an inhibition of cocaine sensitization in rats (Alserda *et al.*, 2013). Furthermore, animals with invalidation of AgRP expression exhibit enhance response towards cocaine intake (Dietrich *et al.*, 2012).

- ***Pomc***: cocaine-SA decreased Pomc mRNA in NAc (only in Fisher rats) (Valenza *et al.*, 2016) as here in our analysis. Pomc is the precursor of β-endorphin. Interestingly, blocking β-endorphin in NAc enhances cocaine-SA responses in rats (Roth-Deri et al., 2004).

- ***Ehmt2*** (Euchromatic Histone Lysine Methyltransferase also known as G9a): Nestler's group characterized the role of this methyltransferase. Recently, a knockdown of this gene in NAc was shown to decrease cocaine-SA and stress-induced reinstatement in rats (Anderson, Sun, et al., 2019).

- ***Gabra1*** (gamma-aminobutyric acid type A receptor alpha1 subunit): even if this subunit is strongly expressed in NAc, short or prolonged withdrawal following cocaine-SA did not affect its expression in NAc of rats (Purgianto et al., 2016). Interestingly, inhibition of accumbal GABAergic neurons attenuated the expression of cocaine CPP in mice (Zhang et al., 2018).

- ***Rxrb*** (Retinoid X receptor**)**: I found only two studies investigating the relationship between retinoid X receptor and cocaine. For instance, cocaine impairs retinoid X receptor signaling in forebrain of mice (Kovalevich *et al.*, 2012). Interestingly, a knockdown of one of the degrading enzymes, *Cyp26b1*, increased cocaine-SA and seeking in rats (Zhang, Kong, *et al.*, 2016).

- ***Grm2*** (glutamate metabotropic receptor 2): mGluR2 agonists are well described as modulator of cocaine intake. For instance, when injected into NAc, they decrease cocaine seeking in rats (Peters & Kalivas, 2006).

- ***Slc18a1*** (solute carrier family 18 member 1 also known as VMAT1): VMAT1 was believed as expressed only in the periphery but recent data indicate that VMAT is functionally expressed in brain (Multani *et al.*, 2013). To my knowledge, there are no studies regarding the relationships between VMAT1 and cocaine intake. Thus, our findings suggest that further studies could investigate that matter.

- ***Oprk1*** (Kappa opioid receptor): Kappa receptor is highly involved in cocaine addiction as this receptor modulates cocaine intake. However, to avoid repetition, the role of Oprk1 is described in our article *"Voluntary cocaine intake modulates mu opioid receptors in the hippocampus"*.

- ***Tacr1*** (Tachykinin Receptor 1 also known as neurokinin 1): I did not find clear investigations regarding the neurokinin 1 receptor, cocaine and NAc. However, an interesting paper found that infusions of the neurokinin 1 receptor antagonist, L-733,060, decreased cocaine-evoked striatal dopamine overflows in rats (Kraft *et al.*, 2001), suggesting a role for neurokinin in addiction. Future studies should investigate whether this receptor or its main endogenous ligand, substance P, are further involved in cocaine addiction.

- ***Enpp1*** (Ectonucleotide Pyrophosphatase/Phosphodiesterase 1): this gene has recently been identified as risk factor for obesity (Mărginean *et al.*, 2019). Giving the similarities between obesity and addiction, this gene deserves further investigation.

In a nutshell, despite our troubles with this transcriptomic analysis, it appears that some of the regulated genes from our analysis have been already described in previous studies which give us some confidence in our results. Thus, investigating further the role of *Slc18a1*, *Tacr1* and *Enpp1* in cocaine effect could be of interest. Despite our careful RNA preparation, we obtained a strong variability among our samples of which we have not identified the cause except maybe the low

number of samples (n=4). Therefore, our future genome-wide experiments, we will include more samples despite the cost of the experiment.

V. Chromosome conformation analysis: troubleshooting with targeting Faah

To better understand the mechanisms at the origin of the mRNA regulations we observed in our study, we decided to investigate the chromosomal interactions. Indeed, such approaches enable to understand the link between nuclear structure and function (Davies *et al.*, 2017).

To do so, we used a chromosome conformation capture approach bridging imaging and molecular technologies. Giving the marked mRNA increase of both CB1 and Faah in the HPC and NAc ***"Article 1**: Voluntary cocaine intake modulates the endocannabinoid system in hippocampus",* we analyzed these potential interactions at the level of both genes, in both structures. The chromosome conformation capture relies on primers targeting specifically a region of interest in the gene body, usually the promotor region. We achieved to target CB1 promoter region as described in our manuscript *"**Article 1**: Voluntary cocaine intake modulates the endocannabinoid system in hippocampus"*. This allowed to obtain an interesting mapping around the promotor region and its interactions with surrounding regions.

However, targeting Faah was more complex. Indeed, as chromosome conformation capture relies on restriction enzymes, one must be careful when designing the primers and ascertain that primers are indeed between the two restriction sites, targeted within a certain base pairs range. With Faah promoter and other gene body regions, we found that we were unable to properly design the primers, fitting these criteria. Nonetheless, we were able to design primers targeting the downstream region of Faah. The results we obtained are represented in **Figure 22**. We found multiple interacting regions with the downstream targeted region. Contrary to CB1, we obtained different patterns between HPC and NAc. Thus, the interpretation is quite difficult as we observed multiple marked peaks. Because of lack of time, as these results were only obtained this summer, we did not pursue further the examination of Faah, because of lack of time. However, we could improve Faah targeting by using other restriction enzymes. Thus, we may be able to design new primers targeting Faah promoter region.

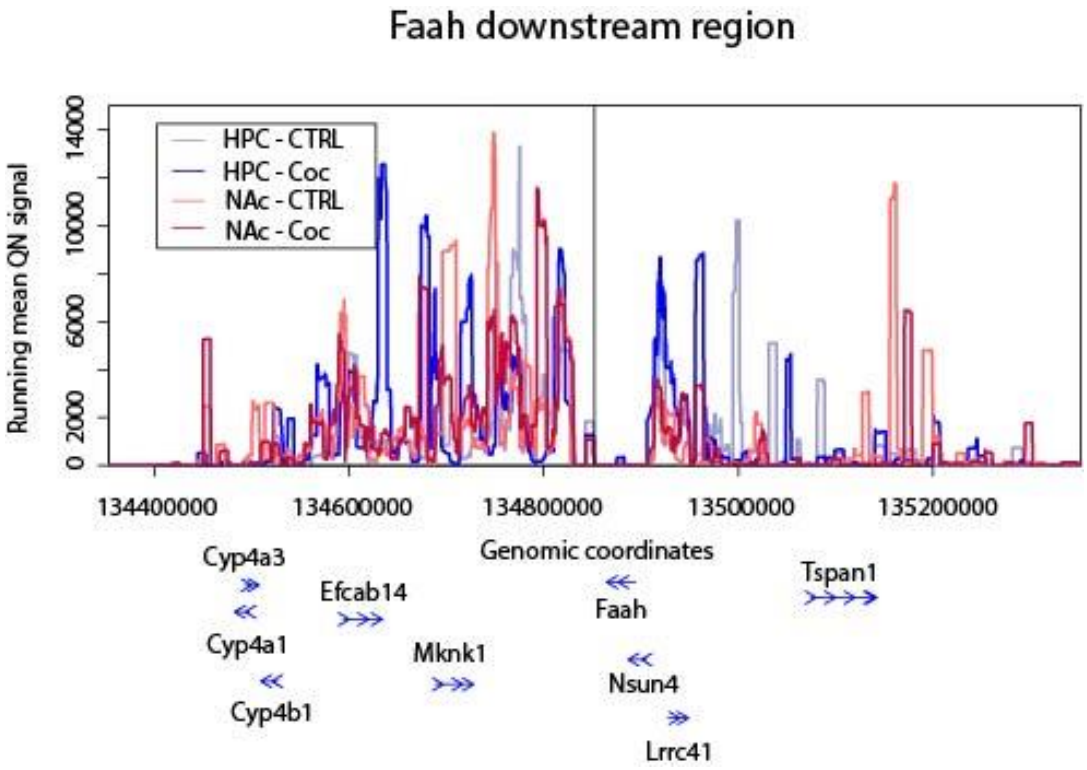

Figure 22: Map representing the interacting chromosomal regions with Faah downstream region. *Faah targeted region is represented by the grey bar in the middle. The peaks represent the intensity of the interaction between the corresponding region and Faah downstream region. HPC and NAc samples (controls (CTRL) and cocaine-SA (Coc)) are represented in blue and red shades respectively.*

Articles

D. Articles

I. Article 1: Cocaine-induced neuroadaptations of the endocannabinoid system in reward-related brain regions: new insights into epigenetic regulations of cannabinoid genes

Many evidences demonstrate the involvement of the ECS in reward, in particular regarding cocaine. In the following section, I will describe the relationships between the ECS and cocaine. Furthermore, to better understand ECS-mediated effects on cocaine intake or seeking, it appears essential to analyze how cocaine modulates the ECS in reward-related brain regions on gene expression, protein and biochemical levels. I summarized these results in a table based on a literature search below.

1. Influence of the ECS on cocaine reward

i. Conditioned place preference

The first clear evidence of CB1-mediated alteration of reward behavior was performed using cocaine-CPP. Chaperon et al conducted two cocaine-CPP experiments (2mg/kg) to evaluate the effect of CB1 blocking (using Rimonabant, an antagonist) on either cocaine-CPP acquisition or expression in rats. Interestingly, when Rimonabant was injected with cocaine during conditioning, animals spent less time in the cocaine-paired compartment (Chaperon *et al.*, 1998). However, Rimonabant did not produce any effects when injected before the final test session (Chaperon *et al.*, 1998) suggesting the ECS is only necessary for the acquisition of a cocaine rewarding behavior. Another study using WT and CB1-KO mice found no differences in a similar cocaine-CPP paradigm but with a higher cocaine dose (20mg/kg) (Martin *et al.*, 2000). As one could have expected similar findings between these two studies, these discrepancies may be explained by: the animal model (rat vs mice), the cocaine dose (2mg/kg vs 20mg/kg) and the potential development of compensatory alterations in the CB1-KO model (Befort, 2015). More recently, Rimonabant mediated decrease of cocaine-CPP acquisition (20mg/kg) was reproduced in mice (Yu *et al.*, 2011). However, Yu et al. also found a decrease of cocaine primed (5mg/kg) reinstatement after 2 weeks of extinction suggesting that CB1 may also be involved in cocaine seeking (Yu *et al.*, 2011). To decipher the role of CB1 in cocaine-CPP, Hu et al. used intra-PFC injection of Rimonabant in mice. Thus, Rimonabant facilitated low dose cocaine-CPP acquisition (2.5, 5 and 10 mg/kg) whereas Rimonabant diminishes cocaine-CPP acquisition at the 10 and 40mg/kg doses (Hu *et al.*, 2015). In

this study, Rimonabant also elicited extinction facilitation. The authors observed similar effects using another CB1 antagonist (1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-*N*-4-morpholinyl-1*H*-pyrazole-3-carboxamide (AM281)) (Hu *et al.*, 2015). Finally, intra-accumbal Rimonabant infusions even promoted conditioned place aversion towards cocaine in a cocaine-CPP paradigm with rats (Ramiro-Fuentes *et al.*, 2010). Altogether, these findings confirmed that CB1 is necessary for the rewarding effects of cocaine. However, deciphering the role of CB1 in other reward-related structures such as the VTA, RMTg, HPC and Amy appears crucial as the two latter studies show different effects of CB1 memory (Ramiro-Fuentes *et al.*, 2010; Hu *et al.*, 2015).

Other studies investigated whether THC and CBD could modulate cocaine-CPP. CBD dose-dependently attenuated cocaine-CPP (10mg/kg) in mice (Luján *et al.*, 2018). Interestingly, both THC and CBD potentiated extinction of cocaine-CPP learning and this effect was not reversed by Rimonabant in rats (Parker *et al.*, 2004). These findings suggested that CB2 could be at the origin of these observations as the ligands used in these studies are not selective agonist **Table 4**. Using a specific CB2 agonist, JWH133, Delis et al. observed a decrease of both acquisition and expression of cocaine-CPP (20mg/kg) in rats (Delis *et al.*, 2017). They also reproduced previous results cited above, with Rimonabant decreasing both acquisition and expression of cocaine-CPP (20mg/kg) (Delis *et al.*, 2017). Interestingly, in an CB2 overexpression (CB2xP) model, CB2xP mice presented cocaine-induced conditioned place aversion (20mg/kg). Overall, studies above demonstrated an involvement of both CB1 and CB2 in cocaine-CPP. Nonetheless, they highlighted the complexity of the ECS in reward modulation. Reports investigating the role of CB2 are still recent and further studies are needed to decipher the role of CB2 in these processes. Finally, modulating the expression of both CB1 and CB2 in specific cell type may bring new insights toward a better understanding of ECS - cocaine reward relationships.

ii. Cocaine sensitization

Another way to study the influence of ECS on cocaine responses was to use behavioral sensitization to cocaine. Arnold et al. treated rats with cannabinoids to modulate behavioral sensitization to cocaine (15mg/kg). Co-administration of both 2-[(1R,2R,5R)-5-Hydroxy-2-(3-hydroxypropyl)cyclohexyl]-5-(2-methyloctan-2-yl)phenol (CP55,940) (agonist) and cocaine diminished locomotor activity but rats still exhibited cocaine sensitization (Arnold *et al.*, 1998). Interestingly, acute cocaine and behavioral sensitization (10mg/kg) induced the same response in both WT and CB1-KO mice (Martin *et al.*, 2000; Corbille *et al.*, 2007). Other studies evaluated whether co-administration of Rimonabant could affect cocaine sensitization (20mg/kg or 15mg/kg) in mice but their results indicated that CB1 was not involved (Lesscher *et al.*, 2005; Gerdeman *et al.*, 2008). When evaluating the effect of THC and THC/CBD, the authors observed that behavioral sensitization (15mg/kg) was neither blocked nor enhanced by cannabinoid pretreatment (Gerdeman *et al.*, 2008) as shown recently in mice (Luján *et al.*, 2018). However,

another report showed that exposure to WIN55,212 in rat adolescence increased cocaine sensitization in adulthood (Kononoff et al., 2018) suggesting that cannabinoids induced long-lasting neuroadaptations increasing the sensitivity of animals towards cocaine rewarding properties. With two cocaine injections separated 1 week apart in mice, 1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-(1-piperidyl)pyrazole-3-carboxamide (AM251) a selective CB1 antagonist, abolished cocaine sensitization (10 or 20mg/kg) when administered upon the first cocaine injection (Corbille *et al.*, 2007). When separated by only 24hr, Rimonabant strongly attenuated behavioral sensitization (10 or 20mg/kg) in rodents (Marinho *et al.*, 2015; Mereu *et al.*, 2015) whereas URB597 (a FAAH inhibitor) facilitates sensitization in mice (Mereu *et al.*, 2015). Noteworthy, despite the moderate involvement of CB1 in cocaine sensitization, Gerdeman et al. hypothesized that CB1 was necessary for behavioral sensitization maintenance. Therefore, after establishing cocaine sensitization (15mg/kg), mice were treated with Rimonabant for five consecutive days before a final cocaine challenge. Here, Rimonabant strongly decreased cocaine sensitization (Gerdeman *et al.*, 2008) and this result was recently confirmed in a similar paradigm in rats (Marinho *et al.*, 2017). A study investigated the specific role of CB1 in NAc by injection of Rimonabant directly in NAc of rats either during induction of cocaine sensitization (10mg/kg) or before the challenge. Interestingly, only the NAc Rimonabant injection in NAc before the challenge diminished the expression of cocaine sensitization (Ramiro-Fuentes & Fernandez-Espejo, 2011).

Regarding CB2, CB2xP mice showed decreased motor response to acute administration of cocaine (10 or 20 mg/kg) and cocaine-induced motor sensitization compared with WT mice (Aracil-Fernández *et al.*, 2012). Interestingly, psychostimulant sensitization was absent in DAT-CB2-KO mice (Canseco-Alba *et al.*, 2019). These findings highlighted a role for CB2 in cocaine sensitization which remained to be deciphered as latter studies showed opposite results.

Overall, reports above highlighted CB1 as involved rather in the expression of cocaine sensitization compare to its induction. Noteworthy, there is a strong lack of evidence regarding the specific role of CB1 in other reward-related regions than NAc and further studies are needed to decipher its implication. Furthermore, while most studies focused on CB1, only few investigated the involvement of others ECS elements such as CB2 or enzymes. Further work is needed to clarify the relationships between the ECS and behavioral sensitization.

iii. Cocaine self-administration

Researches assessed the relationships between cocaine-SA and the ECS in the late 90's. In 1999, Fattore et al. analyzed the effect of a pre-treatment with either WIN55,212, Rimonabant or both, on cocaine-SA in rats. Very interestingly, they not only observed a decrease of cocaine intake in rats pre-treated with WIN55,212 but also this effect was reversed by Rimonabant co-

administration, suggesting that CB1 is necessary for this effect (Fattore *et al.*, 1999). Rimonabant pre-treatment alone did not produce any effects on cocaine intake suggesting that CB1 activation is necessary (Fattore *et al.*, 1999) . De Vries et al. completed these previous results, by demonstrating that the CB1/CB2 agonist, HU210, promotes cocaine reinstatement after extinction in rats (De Vries *et al.*, 2001). This effect was blocked by Rimonabant, suggesting that CB1 was involved and not CB2 (De Vries *et al.*, 2001). Next, they showed that Rimonabant attenuated cocaine and cue-induced reinstatement, but not stress-induced reinstatement (De Vries *et al.*, 2001; Ward *et al.*, 2009). However, Rimonabant itself did not affect cocaine-SA acquisition (De Vries *et al.*, 2001; Lesscher *et al.*, 2005; Xi *et al.*, 2008) nor did AM251 (Xi *et al.*, 2008). On another aspect, a recent study indicated that exposure to WIN55,212 in the adolescence decreased cocaine-SA acquisition in adult rats with short access to cocaine but increased cocaine infusion number in the first hour in rats with long access suggesting a binge intake behavior (Kononoff *et al.*, 2018). This result suggested that cannabinoid-induced long-term neuroadaptations modulate the sensitivity of animals towards cocaine. The difference observed between rats with short or extended-access might be due to a modification of the eCBs tone (see below (Orio et al., 2009)). A first study assessed whether cocaine-SA (0.1mg/kg) would be modulated in CB1-KO mice. They observed no differences between CB1-KO and WT mice on cocaine-SA intake (Cossu *et al.*, 2001). However, Soria et al. obtained an opposite result, with CB1-KO mice exhibiting a lower cocaine-SA intake (0.32, 1, 3.2mg/kg), and a decrease of motivation towards cocaine intake (Soria *et al.*, 2005). Interestingly, the cocaine dose-responses was strongly flattened in CB1-KO mice indicating that the sensitivity of CB1-KO mice could be altered. Rimonabant injections in WT mice or rats mimicked the effects of CB1-KO mice on motivation towards cocaine intake (Soria *et al.*, 2005; Ward *et al.*, 2009), as did AM251 in rats (Xi *et al.*, 2008). The discrepancies between these two CB1-KO studies could be explained by the differences in cocaine concentration (see above). But most importantly, mice were physically restrained in Cossu et al. (tail taped) compare to Soria et al., a stressful condition that could have biased the results (Cossu *et al.*, 2001; Soria *et al.*, 2005). More recently, an elegant study used mouse models where CB1 gene was invalidated in either GABA (GABA-CB1-KO) or glutamatergic (Glu-CB1-KO) neurons in a cocaine-SA paradigm. First, GABA-CB1-KO mice showed a higher sensitivity towards cocaine reinforcement while Glu-CB1-KO mice showed no differences (Martín-García *et al.*, 2016). Nonetheless, Glu-CB1-KO mice exhibited facilitation towards cue-induce cocaine seeking (Martín-García *et al.*, 2016). Thus, GABA-CB1 appeared more involved in cocaine acquisition compare to Glu-CB1, which could control reinstatement processes. This study brought new insights by deciphering the role of CB1 regarding its neuronal expression. A next interesting approach would be to re-express CB1 in specific structures to better understand its specific role (rescues approaches).

Orio et al. performed intra-accumbal injections of Rimonabant in rats with short or long access to cocaine-SA. Rimonabant decreased motivation towards cocaine intake but animals with extended

access were more “sensitive” to Rimonabant, while only the strongest dose affected rats with short access (Orio *et al.*, 2009).

Regarding CB2 involvement in cocaine-SA, the first evidence brought by Gardner’s lab indicated that JWH133, a selective CB2 agonist, dose-dependently inhibited cocaine-SA intake as well as motivation towards cocaine intake (Xi *et al.*, 2011). This effect was reversed by 6-Iodopravadoline (AM630), a selective CB2 antagonist and absent in CB2-KO mice. Interestingly, this effect was mimicked by both intra-nasal and accumbal injections of JWH133 (Xi *et al.*, 2011) and by overexpression of CB2 (Aracil-Fernández *et al.*, 2012). The same group then identified CB2 expression in VTA dopaminergic neurons as stated previously (Zhang *et al.*, 2014).Micro-injections of JWH133 directly into VTA decreased cocaine intake in mice, and this effect was reversed by co-administration AM630 (Zhang *et al.*, 2014).

The situation appeared even more complex when species differences regarding CB2 expression in VTA were demonstrated. JWH133 (20mg/kg) administration did not alter cocaine-SA consumption in rats, but also it enhanced motivation towards cocaine intake in rats (Zhang *et al.*, 2015), an opposite effect from the studies performed in mice. However, an intranasal administration of JWH133 (50µg/side) attenuated motivation toward cocaine intake in rats. The authors argued that enhanced motivation could be a compensatory behavior in response to a decrease in cocaine’s rewarding properties following low doses of JWH133 (Zhang *et al.*, 2015). Using a CB2 antagonist, 5-(4-Chloro-3-methylphenyl)-1-[(4-methylphenyl)methyl]-N-[(1S,2S,4R)-1,3,3-trimethylbicyclo[2.2.1]heptan-2-yl]-1H-pyrazole-3-carboxamide (SR144528), another study found no effect on cocaine-SA intake but a decrease of cocaine-induced reinstatement of cocaine-seeking behavior in rats (Adamczyk, Miszkiel, *et al.*, 2012). Altogether, it is difficult to conclude on the effect of CB2 on cocaine rewarding properties as mainly only one group investigated its effects.

I surprisingly found only a handful studies addressing the relationships between ECS enzymes and cocaine-SA. Using two FAAH inhibitors, phenylmethylsulphonyl fluoride or URB597, both attenuated cue-induced cocaine reinstatement while URB597 also decreased cocaine-induced cocaine-seeking in rats (Adamczyk *et al.*, 2009). In contrast, while intravenous administration of URB597 indeed increased AEA levels, it did not blocked cocaine reinstatement in squirrel monkeys (Justinova *et al.*, 2008)

Finally, recent data indicated that chronic CBD treatment reduced cocaine-SA intake in rats and drastically decreased motivation towards cocaine-SA intake (Luján *et al.*, 2018) whereas acute CBD treatment did not alter cocaine-SA nor cocaine seeking (Mahmud *et al.*, 2017).

In conclusion, studies above strongly suggest that CB1 is rather involved in cocaine seeking than in acquisition whereas CB2 is more involved in cocaine-SA acquisition (but there is still a lack of

studies on the relationships between CB2 and cocaine-SA reinstatement). Nevertheless, as Martin-Garcia et al. demonstrated, the situation is more complex as CB1 is expressed in both GABAergic and glutamatergic neurons (Martín-García *et al.*, 2016). Future studies should target specific neuronal population expressing CB1 to further decipher its involvement in cocaine-SA, even astroglial or mitochondrial CB1 using viral approaches for instance (Hebert-Chatelain *et al.*, 2016; Gutiérrez-Rodríguez *et al.*, 2018). Moreover, there is a surprising strong lack of evidences regarding ECS enzymes in the context of cocaine-SA probably because of how challenging it is to target specifically eCBs enzymes. To conclude, even if CB1 appears as a highly promising target to treat cocaine use, its application in that context is limited by its potential psychiatric side effect, such as anxiety and depression (Moreira & Crippa, 2009).

2. Cocaine-induced modifications of cannabinoid genes, receptors and endocannabinoids levels

i. Cannabinoid receptors

Chronic cocaine injections (15 mg/kg) induced a decrease of CB1 gene expression in ventromedial hypothalamic nucleus and the superficial and deep layers of the cerebral cortex but no changes on CB1 binding were observed (González, Fernández-Ruiz, *et al.*, 2002) **(Table 6)**. Interestingly, in cocaine addicts as well as in chronically cocaine-injected animals (20mg/kg), CB1 was also decreased in PFC and cerebral cortex respectively (Álvaro-Bartolomé & García-Sevilla, 2013) **(Table 6)**.. In adolescent rats, chronic cocaine injections (15mg/kg) briefly increased CB1 expression in PFC, HPC and decreased CB2 expression in PFC (García-Cabrerizo & García-Fuster, 2016) **(Table 6)**. De Fonseca group used a cocaine sensitization protocol and measured both gene and protein expression of ECS elements in PFC, HPC and CRB **(Table 6)**. Briefly, CB1, FAAH and MAGL expression were up-regulated in PFC (Blanco *et al.*, 2014) **(Table 6)**. DAGLα and FAAH expression decreased in HPC (Blanco *et al.*, 2016) and CRB (Palomino, Pavon, *et al.*, 2014) respectively **(Table 6)**. Altogether, studies above indicated that the ECS is strongly affected by chronic cocaine injections regardless of the dose or the paradigm.

In cocaine-SA paradigms, Rivera et al. analyzed specie differences between Lewis and Fisher344 rats on cocaine-induced (1mg/kg) mediated effects on the component of the ECS (CB1, CB2, DAGLα, MAGL, NAPE-PLD and FAAH). Briefly, although there were differences between Lewis and Fisher344 strains, hippocampal CB1 and CB2 were decreased and increased respectively (Rivera, Miguéns, *et al.*, 2013) **(Table 6)**. Regarding enzymes, DAGLα, MAGL and FAAH expression increased in HPC while NAPE-PLD expression decreased, suggesting a modulation of eCBs levels (Rivera, Miguéns, *et al.*, 2013) **(Table 6)**. Following cocaine-SA (0.5mg/kg), CB1 binding was up-regulated in numerous brain areas. Interestingly, most of these neuroadaptations persisted after extinction (Adamczyk, Faron-Górecka, *et al.*, 2012). Only cortical and striatal adaptations occurred

in yoked cocaine animals suggesting that these latter adaptations were not due to motivational processes associated with reinforced responding (Adamczyk, Faron-Górecka, *et al.*, 2012) **(Table 6)**. Cocaine-SA (0.5mg/kg) induced an increase of CB1 only in the group of rats with extended access to cocaine in PFC, NAc and Amy compared to rats with short access to cocaine and saline (Orio *et al.*, 2009). Cocaine-SA in mice strongly increased CB2 gene expression in VTA (Zhang *et al.*, 2017) **(Table 6)**. Finally, Bystrowska et al. observed a decrease of CB1 expression in PFC, DS and Amy following cocaine-SA (0.5 mg/kg). Following extinction, CB1 expression increased in substantia nigra and Amy while CB2 expression decreased in PFC and NAc (Bystrowska *et al.*, 2018) **(Table 6)**. Overall, only few studies investigated the effects of voluntary cocaine intake on the ECS. Most of them focused on a specific structure or CB1. Nonetheless, it appears that CB1 expression is up-regulated following cocaine-SA.

ii. Endocannabinoids

The only way to measure eCBs is to use liquid-chromatography followed by mass spectrometry giving their lipidic nature. Various protocols exist to extract eCBs in the literature that are summarized in part *"**C**: Protocol optimization ; **III**: Mass spectrometry: troubleshooting for detecting and measuring Anandamide in brain samples"* (see review (Buczynski & Parsons, 2010)). Noteworthy, all studies below used a methanol/chloroform extraction method to measure eCBs levels. However, methanol converts 2-AG into 1-AG. Thus, all findings below included 1-AG in their analysis which could have biased the precision of 2-AG measurements in their respective analysis.

A first study using chronic cocaine injections (15 mg/kg) revealed a decrease of 2-AG levels in limbic forebrain without any changes in several other brain regions (González, Grazia Cascio, *et al.*, 2002). Microdyalisates measures of eCBs in NAc shell of rats self-administering cocaine (0.25 mg/0.1 ml) showed no changes (Caillé *et al.*, 2007) **(Table 6)**. On the opposite, another group found that in rats with short and extended access to cocaine-SA (0.5mg/kg), eCBs levels were decreased in NAc shell of the short access group (Orio *et al.*, 2009) **(Table 6)**.

Bystrowska et al. used cocaine-SA (0.5mg/0.1ml) with an additional yoked (non-contingent control) group and measured eCBs levels after of cocaine-SA or extinction. Most changes occurred after extinction and are summarized in (Bystrowska *et al.*, 2014) **(Table 6)**. A recent study from the same group using cocaine-primed reinstatement (10mg/kg) following cocaine-SA (0.5mg/0.1ml) showed many changes on eCBs levels in several reward-related brain regions in rats with voluntary intake and yoked animals which are summarized in **Table 6**. Briefly, the authors observed an increase of eCBs in HPC with a strong increase of AEA in PFC and decrease in NAc (Bystrowska *et al.*, 2019) **(Table 6)**. Studies from Bystrowska et al. suggested that eCBs

level changes are not only structure specific but also that they mainly occur following extinction and reinstatement instead of shortly after cocaine intake.

Overall, concluding on cocaine-mediated changes on eCBs is troublesome as only few studies investigated that matter and used different protocols. Moreover, the discrepancies between voluntary and passive cocaine intake protocols highlight cocaine-mediated effects may be different whether passive or voluntary intake is involved, suggesting distinct profile of adaptations.

ECS element	Passive cocaine intake	Voluntary cocaine intake	Extinction of cocaine seeking	Reinstatement of cocaine seeking
CB1	**↑ PFC, HPC (Blanco *et al.*, 2014, 2016)** **↓ Cortex, HYP (González, Fernández-Ruiz, *et al.*, 2002)** **↑ PFC, VTA (Blanco *et al.*, 2014; García-Cabrerizo & García-Fuster, 2016; Bystrowska *et al.*, 2018)** **↓ PFC, BLA (Alvaro-Bartolome & Garcia-Sevilla, 2013; Bystrowska *et al.*, 2018)**	**↑ Cortex, PFC, Septum, NAc, DS, HPC, VTA, PAG (Adamczyk, Faron-Górecka, *et al.*, 2012; Bystrowska *et al.*, 2018)** **↓ PFC, BLA, DS, HPC (Rivera, Miguéns, *et al.*, 2013; Bystrowska *et al.*, 2018)** **↑ NAc, Amy (Orio *et al.*, 2009) after extended access only**	**↑ Cortex, cingulate cortex, Septum, Striatum, NAc, HPC, PAG (Adamczyk, Miszkiel, *et al.*, 2012) Binding** **↑ Substantia nigra (Bystrowska *et al.*, 2018)** **↓ Globus pallidus (Bystrowska *et al.*, 2018)**	**↑ PFC, Lateral septum (Bystrowska *et al.*, 2019)** **↓ VTA (Bystrowska *et al.*, 2019)**
CB2	**↑ NAc shell (Bystrowska *et al.*, 2018)** **↓ PFC (García-Cabrerizo & García-Fuster, 2016)**	**↑ VTA** (Zhang *et al.*, 2017) **↑ HPC (Rivera, Miguéns, *et al.*, 2013)** **↓ BLA (Bystrowska *et al.*, 2018)**	**↓ PFC, NAc core, Globus pallidus (Bystrowska *et al.*, 2018)** **↑ Cortex, cingulate cortex, Septum, Striatum, NAc, HPC, PAG (Adamczyk, Miszkiel, *et al.*, 2012) Binding**	**↑ PFC, Lateral septum (Bystrowska *et al.*, 2019)**
NAPE-PLD	**↑ PFC (Blanco *et al.*, 2014)**	**↓ HPC (Rivera, Miguéns, *et al.*, 2013)**		
FAAH	**↑ HPC, PFC (Blanco *et al.*, 2014, 2016)** **↑ PFC (Blanco *et al.*, 2014)** **↓ CRB** (Palomino, Pavon, *et al.*, 2014)	**↑ HPC (Rivera, Miguéns, *et al.*, 2013)**		

DAGLα	**↓ HPC (Blanco *et al.*, 2016)** **↓ HPC (Blanco *et al.*, 2016)**	**↑ HPC (Rivera, Miguéns, *et al.*, 2013)**		
MAGL	**↑ PFC (Blanco *et al.*, 2014)**	**↑ HPC (Rivera, Miguéns, *et al.*, 2013)**		
AEA	**↓PFC, NAc, CRB (Bystrowska *et al.*, 2014)**	**↓CRB (Bystrowska *et al.*, 2014)** **↓NAc shell (Orio *et al.*, 2009)**	**↑ CRB (Bystrowska *et al.*, 2014)** **↓ Frontal cortex, NAc, HPC (Bystrowska *et al.*, 2014)**	**↑ PFC, HPC (Bystrowska *et al.*, 2019)** **↓Frontal cortex, NAc, DS, CRB (Bystrowska *et al.*, 2019)**
2-AG	**↑ Frontal cortex, HPC (Bystrowska *et al.*, 2014)** **↓ Limbic forebrain (González, Fernández-Ruiz, *et al.*, 2002)**	**↑ Frontal cortex, CRB (Bystrowska *et al.*, 2014)** **↓ DS, HPC (Bystrowska *et al.*, 2014)** **↓NAc shell (Orio *et al.*, 2009)**	**↑ PFC, NAc (Bystrowska *et al.*, 2014)** **↓ DS, HPC, CRB (Bystrowska *et al.*, 2014)**	**↑ HPC, NAc (Bystrowska *et al.*, 2019)** **↓ Frontal cortex, CRB (Bystrowska *et al.*, 2019)**

Table 6: Summary of cocaine-induced adaptations on the ECS in brain. *Legend:* ***gene expression, eCBs levels, eCBs levels by microdialysis; protein level by immunostaining; ligand binding.***

As presented above, many studies investigated cocaine-induced neuroadaptations of the ECS to better understand the relationships between cocaine and the ECS. However, these reports exhibit many discrepancies and did not necessarily used an approach with voluntary cocaine intake. In the following paper, we used a voluntary cocaine intake paradigm to assess the impact of cocaine on the ECS in many reward-related regions. We focused on transcriptional and protein alterations, eCBs levels and epigenetic adaptations.

Voluntary cocaine intake modulates the endocannabinoid system in hippocampus

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Key words: CB1 cannabinoid receptor, cocaine, hippocampus, gene expression, epigenetic

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Abbreviations:

Amy: amygdala; Cocaine-SA: cocaine self-administration; CPP: compartment place preference; DS: dorsal striatum; ECS: endocannabinoid system; GPCR: G protein coupled receptor; HPC: hippocampus; LTP: long-term potentiation; PFC: prefrontal cortex; NAc: Nucleus Accumbens; VTA: ventral tegmental area.

Abstract

Drug addiction is a complex pathology inducing long-term neuroplasticity. Understanding the neurochemical mechanisms underlying the reinforcing effects of drugs of abuse is critical to reduce the burden of drug addiction in society. The endocannabinoid cannabinoid system is strongly involved in the modulation of drug reward. The endocannabinoid comprises endocannabinoids, enzymes for their synthesis and degradation, and two well characterized receptors, CB1 and CB2, both coupled to Gi/Go proteins. Blocking CB1 decreases cocaine-seeking while CB2 activation reduces cocaine intake. However, the underlying mechanisms of this modulation remain poorly understood. In this study, we investigated whether chronic cocaine treatment induces long-term adaptations including transcriptional modifications and their potential associated epigenetic processes. We first examined gene expression following either intraperitoneal injections (20mg/kg, 10 days) or intravenous cocaine self-administration (0.33mg/kg, FR1, 10 days) in reward related rat brain regions. Interestingly, despite almost no regulations induced by cocaine injections, we found an increase of CB1 gene expression in several structures with cocaine self-administration, with a marked increase in the hippocampus. With GTPYS binding, immunofluorescence and western blot, we demonstrated an enhancement of CB1 receptor activity without protein expression regulation in the hippocampus following cocaine self-administration. Endocannabinoid levels measured by mass spectrometry were specifically increased in the hippocampus. Chromatin immunoprecipitation followed by qPCR revealed, in the hippocampus, that histone modifications (H3K4Me3 and H3K27Ac) were enriched by cocaine-SA at endocannabinoid system genes. Finally, using chromosome conformation capture, we show that cocaine self-administration induces interaction changes at CB1 gene promoter locus in both nucleus accumbens and hippocampus. Our data suggest a key role for the hippocampus in endocannabinoid system adaptations following voluntary cocaine intake, suggesting a major role for the endocannabinoid system in reward associated memories.

Introduction

Cocaine addiction is a chronic disease characterized by compulsive drug use despite negative consequences, craving for cocaine and high probability of relapse even after prolonged periods of abstinence (American Psychiatric Association, 2013). Neuroplasticity alterations are strongly involved in the disease, whereby altered gene expression impacts neuronal function and subsequent behavior (McClung & Nestler, 2008). Cocaine causes widespread gene expression changes in brain reward-related regions, notably in nucleus accumbens (NAc), alterations have been widely described (Russo *et al.*, 2010). Among the neurobiological mechanisms involved in addictive behaviors, epigenetic processes are crucial effectors of the long-term adaptations produced by drugs of abuse. Epigenetic mechanisms such as histone tail modifications, DNA methylation (DNAm) or ncRNA either control chromatin accessibility (Engmann *et al.*, 2017) or regulate gene expression, thereby altering the transcriptional activity of genes (Nestler & Lüscher, 2019). Through next generation sequencing, studying the epigenome in addictive behaviors is highly promising but turned out to be far from over (De Sa Nogueira *et al.*, 2019).

Interestingly, the endocannabinoid system (ECS) modulates drug reward (De Vries *et al.*, 2001; Soria *et al.*, 2005; Xi *et al.*, 2011; Zhang *et al.*, 2014). Its neuromodulators named endocannabinoids (eCBs), the anandamide (AEA) and 2-arachidonoylglycerol (2-AG), are released by a « on demand » mechanism through a calcium influx resulting from voltage-gated calcium channels depolarization (Wilson & Nicoll, 2001). Both are lipids acting as retrograde messengers onto two cannabinoid receptors, cannabinoid receptor 1 (CB1R) and 2 (CB2R) which are Gi/o-protein coupled receptors and therefore negatively regulate adenylate cyclase activity (Pertwee, 2006b). CB1R is highly expressed in axons terminals in both periphery and nervous central system therefore its role in the regulation of neurotransmitter release has been widely described (Panagis *et al.*, 2014; Befort, 2015; Zimmer, 2015). CB1R is also functionally expressed on astrocytes which modulates glutamate release (Navarrete & Araque, 2008, 2010). Recently, CB2R expression has been described in brain regions such as the hippocampus (HPC), striatum and thalamus (Gong *et al.*, 2006; Onaivi *et al.*, 2006; Li & Kim, 2015; Stempel *et al.*, 2016). Most importantly, its expression was recently described on the soma of dopaminergic neurons in ventral tegmental area (VTA) (Zhang *et al.*, 2014; Zhang, Gao, *et al.*, 2016). In 2007, a cannabinoid receptor interacting protein (CRIP1a encoded by *Cnrip1*) was discovered (Niehaus *et al.*, 2007). CRIP1a not only modulates CB1R signaling (Niehaus *et al.*, 2007; Smith *et al.*, 2015; Guggenhuber *et al.*, 2016) but also may reduce CB1R endocytosis (Mascia *et al.*, 2017). Similarly to CRIP1a, another CB1R interacting protein was found in 2005, Src homology 3-domain growth factor receptor-bound 2-like interacting protein 1 (SGIP1) (Trevaskis *et al.*, 2005). Recent findings

indicate that SGIP1 could also modulates both CB1R signaling and endocytosis (Hájková *et al.*, 2016).

Both cannabinoid receptors modulate cocaine intake. CB1R agonist, HU210, promotes the reinstatement of cocaine-seeking while a selective CB1R antagonist, SR141716 (Rimonabant), dose-dependently decreases this behavior (De Vries *et al.*, 2001). Moreover, CB1R-KO mice are less prone to self-administer cocaine (Soria *et al.*, 2005). Recent studies indicate that a selective CB2R agonist, JWH133, diminishes voluntary cocaine intake and attenuates cocaine-enhanced extracellular levels of dopamine when injected in NAc or VTA while the selective CB2R antagonist, AM630, prevent these effects (Xi *et al.*, 2011; Zhang *et al.*, 2014; Zhang, Gao, *et al.*, 2016). Moreover, CB1R expressed in GABA neurons regulates sensitivity to cocaine, while CB1R expression in glutamatergic neurons modulates associative learning processes (Martín-García *et al.*, 2016). Such discrepancies make difficult to understand how the ECS is involved in cocaine intake. To better understand the involvement of the ECS in cocaine reinforcement, several studies investigated ECS modifications mostly after passive cocaine intake. For instance, using a cocaine sensitization protocol with mice, De Fonseca group showed an increase of CB1R gene expression in prefrontal cortex (PFC) and HPC (Blanco *et al.*, 2014, 2016) with no effects in cerebellum (Palomino, Pavon, *et al.*, 2014). Interestingly, using cocaine self-administration (cocaine-SA) with two different rat strains, they observed a decrease of CB1R immunoreactivity in few hippocampal areas of cocaine-SA rats (Rivera, Miguéns, *et al.*, 2013) in opposite to another study (Bystrowska *et al.*, 2018).

Regarding eCBs levels, a chronic cocaine passive treatment lowered 2-AG levels in the limbic forebrain while AEA levels remained unchanged (González, Fernández-Ruiz, *et al.*, 2002). Only few studies, investigated ECS alterations after cocaine-SA. Microdyalisate measurements showed no changes of extracellular endocannabinoids levels in NAc shell after chronic cocaine-SA (Caillé *et al.*, 2007). More recently, a chronic cocaine-SA treatment elevated 2-AG levels in frontal cortex, cerebellum and lowered levels in HPC and striatum whereas AEA levels were decreased in the cerebellum. After an extinction training, 2-AG levels were increased in PFC, NAC and decreased in HPC, dorsal striatum (DS) and cerebellum whereas levels of AEA were increased in cerebellum and decreased in frontal cortex, HPC and NAc (Bystrowska *et al.*, 2014). Understanding, the involvement of eCBs in addictive behaviors is crucial as the role of 2AG in dopamine modulation has been demonstrated (Wang *et al.*, 2015).

Not only results from the literature are still mixed but most of studies above used passive cocaine intake and did not investigate voluntary cocaine intake effects in brain reward-related regions. This issue is a great matter as reproducing voluntary cocaine-induced modifications using

passive cocaine intake is not ascertained. For instance, persistent LTP in VTA is induced by cocaine-SA but not passive cocaine intake (Chen *et al.*, 2008).

To explain transcriptional regulations of CB1R, only few studies investigate epigenetic modifications behind CB1R gene expression. For instance several studies explored DNAm changes at CB1R gene promoter (Wang *et al.*, 2008; Hong *et al.*, 2015; Mancino *et al.*, 2015; D'Addario *et al.*, 2017; Almeida *et al.*, 2019) as well as histone modification at CB1R gene locus (Subbanna *et al.*, 2014; Lomazzo *et al.*, 2017; Almeida *et al.*, 2019; Jiang *et al.*, 2019). Further studies are needed to explore which epigenetic modifications are crucial at CB1R gene locus.

To better understand the relationship between cocaine and the ECS, we investigated how cocaine-SA modulates the ECS. We describe gene expression modifications of ECS elements, alterations of CB1R expression and functionality along with a modification of eCBs levels in many brain reward-related structures. We previously reported that cocaine induced the expression of epigenetic factors like MeCP2 (methyl CpG binding protein 2) and HDAC2 (histone deacetylase 2) in several rat brain regions including in NAc (Cassel *et al.*, 2006; Host *et al.*, 2011). Therefore, we also investigated how cocaine regulates ECS genes by targeting histones modifications, acetylation of lysine 27 of histone 3 (H3K27Ac) and trimethylation of lysine 4 of histone 3 (H3K4Me3). Finally, to better grasp the involvement of these modifications in CB1R gene expression, we used circularized chromosome conformation capture at CB1R gene promoter locus.

Materials and Methods

Subjects

Male Wistar rats (Janvier, France) weighting 250–300 g at the beginning of the tests were habituated for two weeks to housing conditions in a temperature and humidity-controlled environment with a reverse 12 h light/dark cycle (lights on at 7:00 PM). For intraperitoneal studies, animals were kept in a regular 12h light/dark cycle. Rats were group housed 5 animals/cage in standard cages with *ad libitum* access to food and water until surgery and then single-housed for cocaine-SA experiments. All experimental procedures were performed according to the European Union laws for animal studies and approved by the institutional ethics committee CREMEAS (Comité d'Éthique pour l'Expérimentation Animale de Strasboug, France) #165. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Cocaine passive injections

Three experimental groups were constituted (n=8-12 animals/group). Two groups were injected with either NaCl 0.9% or with 20 mg/kg of cocaine hydrochloride (1164500, Cooper, Melun, France) solution adjusted with NaCl 0.9% once a day for 10 days. The last group was treated with an acute cocaine injection with 20mg/kg after chronic treatment with NaCl 0.9%. Brain extraction was performed 24hr after last injection by giving animals an overdose of pentobarbital 82,20mg/kg i.p followed by decapitation.

Cocaine self-administration procedure

Intravenous catheterization procedure was performed as previously described (Fonteneau *et al.*, 2017). Briefly, rats were anesthetized by the i.p. injection (1 ml/kg) of a mixture containing 90mg/kg of ketamine (Imalgene 1000®, Centravet, France) and 10mg/kg of xylazine (Rompun®,Centravet), prior to surgical implantation of a chronic indwelling catheter in the right jugular vein. The silicone catheter (Silastic®, Plastics One, Roanoke, VA, USA), was fitted to a 23-gauge guide cannula that was bent at a right angle and then embedded in dental cement on a circular 2.5 cm mersilene mesh base. A discrete incision was performed onto the jugular vein; the heparinized catheter was immediately inserted 3.6 cm into the vein and anchored with suture. Catheters were flushed daily with 150 µl saline solution containing 100 U/ml heparin and 50 mg/ml ampicillin to prevent clotting and infection, respectively.

Drug self-administration was performed as previously described (Romieu *et al.*, 2011; Fonteneau *et al.*, 2017) in dark operant chambers (30 × 30 × 30 cm) located in a sound-attenuated room. Briefly, a computer driven syringe pump (Imetronic, Pessac, France) activated a 10 ml syringe and pushed fluid into Silastic® tubing connected to the rat through its externalized 23-gauge guide cannula. Each chamber was equipped with two 2.5 cm-diameter holes on the same wall, located 4 cm above the floor; one was selected as the active hole for delivering the reinforcer and the other as the inactive hole. Disruption of an infrared photobeam in each hole (nose-poke) was detected using a digital input card (DIO-24; National Instrument, Austin, TX, USA) and homemade LabView software (National Instrument). Nose-pokes into both holes were recorded. Nose-pokes into the inactive hole had no programmed consequence. Nose-pokes into the active hole triggered the intravenous (i.v.) delivery of a 0.33 mg/kg dose of cocaine hydrochloride (60 µl over 2 sec) under the control of the computer. A 5 sec flashing light, located 8 cm above the active hole, was paired contingently with the delivery of cocaine solution adjusted with NaCl 0.9% . Injection persisted for 5 s, followed by a white light illuminating the chamber for 35 sec. A 40 sec-time-out period began simultaneously to the cocaine injection. No cut-off was applied concerning the number of self-infusions the rat was able to perform during the session. A fixed-ratio 1 (FR1) cocaine self-administration paradigm was carried out for 10 days during daily 2h sessions. 24hr after last session, animals were given an overdose of pentobarbital (41mg/kg, i.v) followed by decapitation to perform brain extraction. We were careful to execute all extraction procedures in

less than 30min to avoid increased levels of AEA (Schmid *et al.*, 1995). PFC, NAc, DS, LH, dorsal HPC, Amy, VTA and RMTg were microdissected and samples were immediately frozen on dry ice and kept at -80°C.

Quantitative real-time PCR

After brain extraction brain structures of interest were collected by punches. Samples were immediately frozen on dry ice and kept at -80°C. Samples (n=4-11rats/group) were processed to extract total RNA using Ribozol (VWR) according to manufacturer's instructions. RNA quality (260/280 ratio: 1.8–2) and quantity was measured with a NanoVue™ (GE healthcare) spectrophotometer. Reverse transcription to obtained cDNA was performed on 750 ng of total RNA in a 20 µL final volume, with iScript (iScript™ cDNA Synthesis Kit, Biorad, France). Real-time PCR was performed using a CFX96 Touch™ apparatus (Biorad, France) and Sso Advanced™ Universal SYBR Green supermix (Biorad, France) in a final volume of 15 µl. Thermal cycling parameters were 30 sec at 95°C followed by 40 amplification cycles of 5 sec at 95°C and 45 sec at 60°C. Primer sequences for all tested genes are given in **supplemental table 1**. Expression levels were normalized to *rplp0* housekeeping gene levels and compared between controls and treated samples using the 2-ΔΔCt method (Livak & Schmittgen, 2001).

CB1 Immunofluorescence

Animals were slowly (10ml/min) transcardially perfused with saline during 5min followed by 4% paraformaldehyde during 15min. Brains were removed, kept overnight at 4°C in 15% sucrose, frozen in isopentane at -40°C, and stored at -80°C. Brains were cut in sections (30µM) by cryostat. 6 sections of a same brain structure/animal were first incubated with a primary antibody anti-CB1 (1:1000; Frontiers; Af380). Sections were then successively incubated with a donkey anti-rabbit IgG, (Alexa fluor594, 1:500 dilution, A21207, Biorad, France). Sections were then incubated in DAPI (1:2000) to label nuclei and the slides were coverslipped with Mowiol (Calbiochem, MA, USA). Staining was observed under a fluorescent binocular microscope (ZEISS, Apotome). Density measurement of CB1 in structures of interest was achieved using the ImageJ 1.43 software (NIH, MA). The density measures were calculated from the fluorescence mean minus background fluorescence mean. For each measure, were performed on 6 sections from each rat bilaterally. Expression was estimated in subregions of the NAc (core (NAcCo) and shell (NAcSh)), DS (dorso-lateral (DLS) and dorso-median (DMS) and HPC (CA1, CA2 and dentate gyrus (DG)).

Mass spectrometry

Preparation of Tissues

PFC, HPC, NAc and DS tissues were sonicated with a Vibra Cell apparatus (2 times 5 s, 90W; Sonics, Newtown, U.S.A.) in 200 µl of H_2O. The homogenate was centrifuged (20,000g, 30 min, 4°C) and the supernatant was recovered. Protein concentration was determined using the Bradford method (Protein Assay, Bio-Rad, Marnes-la-Coquette, France). 150 µl of the supernatant were taken and mixed with 50 µl of acetonitrile (ACN) 100% containing known fixed amounts of deuterated internal standards containing 400.26 pmol of D8-2AG (ref sc-480539; Santa Cruz, Heidelberg, Germany) and 100.15 pmol of D4-AEA (Tocris/Biotechne, Lille, France). The addition of heavy compounds allows to perform a quantification using isotopic dilution. The sample was centrifuged (20,000g for 30 min, 4°C) and the supernatant was collected and evaporated to dryness. Samples were re-suspended in 20 µl of ACN 30% / H_2O 69.9% / formic acid 0.1% (v/v/v);

LC-MS/MS Instrumentation and Analytical Conditions

Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, USA) coupled with a triple quadrupole Endura mass spectrometer (Thermo Scientific). The system was controlled by Xcalibur v. 2.0 software (Thermo Electron). Samples (3µl) were loaded onto a microbore C18 ODS column (1x100 mm, 3 µm UniJet microbore ODS, ref MF8949, BioAnalytical Systems Inc., West Lafayette, U.S.A.) heated at 40°C. The presence of 2-AG, AEA, D8-2-AG and D5-AEA was studied using the multiple reaction monitoring mode (MRM). Elution was performed at a flow rate of 50 µl/min by applying a linear gradient of mobile phases A/B. Mobile phase A corresponded to ACN 1% / H_2O 98.9% / formic acid 0.1% (v/v/v), whereas mobile phase B was ACN 99.9% / formic acid 0.1% (v/v). The gradient used is detailed in **supplemental table 2**.

Electrospray ionization was achieved in the positive mode with the spray voltage set at 3,500 V. Nitrogen was used as the nebulizer gas. Desolvation (nitrogen) sheath gas was set to 10 Arb and Aux gas was set to 5 Arb. The Ion transfer tube was heated at 287°C. Q1 and Q2 resolutions were set at 0.7 FWHM, whereas collision gas (CID, argon) was set to 2 mTorr. Identification of the compounds was based on precursor ion, selective fragment ions and retention times obtained for 2-AG, AEA, D8-2-AG and D5-AEA. Selection of the monitored transitions and optimization of collision energy and RF Lens parameters were manually determined (see **supplemental table 3**). Qualification and quantification were performed in MRM mode. Quantification was obtained using Quan Browser software (Thermo Scientific). For tissues and fluids, alkaloids were quantified using calibration curves of external standards added to brain extract of naive mice and submitted to the same procedure described for respective fluids and tissue recovery. All amounts of endocannabinoids measured in samples fit within the standard curve limits, with typical analytical ranges (the range of amounts that can be accurately quantified) from 1 fmol – 100 pmol to 150 fmol – 100 pmol. Precision (CV% between repeated injections of the same sample) values were <1% for same-day measurements and <5% for inter-

day measurements. sThe amount of 2AG (nmol) and AEA (pmol) observed were normalized according to protein concentrations (mg).

Agonist-stimulated [^{35}S]-GTPγS binding assay

Arachidonyl-2'-chloroethylamide (ACEA), a potent and highly selective CB1R agonist (Luszczki *et al.*, 2006), was used in [^{35}S]-GTPγS binding assay to measure G protein activation following CB1 receptor stimulation. Brain structures of interest were subjected to GTPγS binding in triplicate in 96 deep-well plates. Tissues were homogenized in sucrose 0.25M then centrifuged at 1100g (4°C) for 10 min. Supernatant were collected and centrifuged at 30000g (4°C) for 30 min. Pellets were then homogenized in sucrose 0.32M and subjected to Bradford analysis for total protein concentration, and then stored at −80 °C. The assay was initiated by the addition of cell membranes (50 µg) to the assay buffer (50mM Tris-HCl pH 7.4; 3 mM MgCl2; 0.2mM EGTA; 100mM NaCl) containing 0.1nM [^{35}S]-GTPγS (NEG030H, PerkinElmer, Courtaboeuf, France), 30 µM GDP and ACEA (10^{-10}M to 10^{-5}M) for 1 h at 25 °C. Radioactivity was detected on a Top-Count scintillation counter (PerkinElmer, Billerica, MA, USA). Basal [^{35}S]-GTPγS binding was determined in the absence of agonist, and non-specific binding by replacing [^{35}S]-GTPγS by 10 µM of non-radiolabeled GTPγS. Stimulated specific binding was converted in percentage of basal specific binding, defined as 100%. Data were analyzed using Prism 6 Graphpad sofware. Four to seven independent assays were performed on three distinct membrane preparations per group in triplicate. Stimulation (%), EC50s and IC50s were calculated for each experiment and averaged.

Western blot

A fraction of samples homogenized in sucrose 0.25M for Agonist-stimulated [^{35}S]-GTPγS binding assay was taken and identical amount of proteins (20 µg) were separated on a stain free polyacrylamide gel 4-15% (Biorad, France) and proteins were transferred onto a PVDF membrane (Biorad, France). Resulting blots were then blocked in PBS-I-block (Tropix, Applied Biosystems), 0.1% Tween 20 buffer for 1h. An overnight incubation at 4°Cs with CB1R antibody was processed (anti-CB1R antibody 1:2000 (Cayman 10006590). Blots were washed and then incubated in biotinylated secondary goat anti-body (1:50 000) for 1h at room temperature (Jackson ImmunoResearch Laboratories, Inc). Antibody binding was revealed by chemiluminescence (ECL Prime, GE healthcare, Piscataway, NJ, USA) detected using the ChemiDoc Imager (Biorad, France). Normalization was performed using stain free (total protein) (Gürtler *et al.*, 2013).

Chromatin immunoprecipitation

Samples were prepared as previously described (Caputi *et al.*, 2014) with minor modifications. Frozen tissues were ground on carbonic ice before fixation for 8 min with 1% formaldehyde and then quenched with glycine (0.125M) for 5 min. Tissue fragments were washed

with cold phosphate-buffered saline supplemented with protease inhibitors (#4693132001, Roche, France). Tissues were then homogenized in 900µl of sonication buffer (0.5% SDS, 10mM EDTA, 50mM Tris-HCl, pH8) and lysates were sheered using a diogenode bioruptor XL at 4°C, at high sonication intensity for 30s ON / 30s OFF for 50 min, to obtain DNA fragments <600 bp. Next, samples were centrifuged at 20000g (4°C) for 20min. Protein A magnetic beads (Dynabeads, Invitrogen) were coated with the respective antibody of interest (H3K4Me3, ab8580; H3K27Ac, ab4729, Abcam, France) at 4°C overnight on a rotator. Following washing of the magnetic beads and associated immune complex, they were added to 200µl of sheared chromatin for histone ChIPs and incubated overnight at 4°C on a rotator. 20µl (10%) of each sample of sheared chromatin was used as input controls. Samples were washed with RIPA buffer (HEPES-KOH 50mM pH7.5, LiCl 500mM, EDTA 1mM, NP-40 1%, Na-Deoxycolate 0.7%) and elution buffer (SDS 1%, $NaHCO_3$ 100mM) on the next day. Reverse cross-linking was performed at 65°C overnight, and proteins and RNA were removed with proteinase K and RNAse A (ThermoFisher, USA) respectively. DNA was purified using a DNA mini elute kit (Qiagen, France). Additionally, a negative control without IgG was performed to test for nonspecific binding. Primers are indicated in **Table 1**.

Circularized chromosome-conformation capture (4C)

4C-seq was performed as previously described with slight modifications (van de Werken *et al.*, 2012). Briefly, frozen NAc (4 12-gauge punches of whole NAc/rat) and whole dissected dorsal HPC/rat from 2 rats were homogenized and cross-linked with 2% formaldehyde and nuclei were extracted following a classical hypotonic shock protocol. Purified nuclei were digested overnight with the 1st restriction enzyme (DpnII) and posteriorly subjected to an over-night ligation. To validate each step an agarose gel was run in order to observe a shift between the undigested, digested and ligated conditions. Subsequently, chromatin was de-crosslinked and purified after proteinase K and RNAse A treatment. A second restriction was performed overnight using CspIA as a 2nd restriction enzyme followed by a final overnight ligation and DNA purification. The resultant 4C DNA template was used to generate 4C-seq libraries by performing a PCR with target-specific designed primers **(supplemental table 1)**. For primer design, a region surrounding the TSS of the gene of interest (+/- 2 kb) was retrieved and primers were designed for regions that fulfilled the following criteria: distance between DpnII restriction site and the consecutive Csp6I restriction site > 350 bp; distance between DpnII restriction site and the following DpnII restriction site after Csp6I > 500 bp and < 1500 bp. A primer validation step was included to verify primers specificity. Finally, generated libraries were then purified with SPRI select beads (Beckman) to discard primer dimer DNA products and 4C-seq DNA template were quantified using Bioanalyzer and pooled equimolarly for sequencing using 50bp single-end Hiseq 4000 sequencer of the Genomeast platform (IGBMC).

The analysis was performed using a custom perl script based on analysis described in (van de Werken *et al.*, 2012) and (Sexton *et al.*, 2012) papers. Resulting reads from 4C-seq sequencing were de-multiplexed in individual fastq files using sabre tool (https://github.com/najoshi/sabre, version 1.0) according to the reading primer of each bait. Fastq reads were then filtered to remove those not containing the 1st restriction enzyme sequence and aligned using Bowtie to the rat Rn6 genome (Langmead *et al.*, 2009). Mapped regions were assigned to a fragment-end coordinate generated by the *in-silico* digestion of the reference genome using the primary and secondary restriction enzymes recognition sequences (DpnII and Csp6I respectively). To allow for data comparison among different conditions, quantile normalization was performed using limma R package (Ritchie *et al.*, 2015) and resulting bedGraphs were observed using an in-house R script. Fragments were clustered by five for the deseq2 analysis (Love *et al.*, 2014) to allow a better data interpretation.

Statistical analysis

All results are expressed as mean ± S.E.M. Data from intraperitoneal injections was analyzed using one-way ANOVA (treatment). Student-Newman-Keuls *post hoc* test was applied when required. All data from experiments conducted after cocaine self-administration for each structure were analyzed student t-test. Significance was set at $p \leq 0.05$.

Results

Transcriptional changes of the ECS induced by intraperitoneal cocaine injections

To test whether cocaine modulates the ECS in brain reward-related regions we first performed a gene expression analysis of *Cnr1*, *Cnr2* and *Cnrip* in PFC, NAc and DS from cocaine and saline-treated rats through intraperitoneal injections. After either acute or chronic cocaine treatment **(Figure 1A)**, we found that the ECS was mostly unaffected by cocaine treatment compared to saline treated rats **(Figure 1B)**. *Cnr1* expression did not differ between control and cocaine treated animals [***Cnr1***: (*PFC:* F (2, 32) = 0.2118, *p* = 0.8102), (*NAc*: F (2, 30) = 2.532, *p* = 0.0964), (*DS*: F (2, 31) = 4.159, *p* = 0.0251)]. In DS, chronic cocaine decreased *Cnr1* expression compared to the acute group (**Figure 1B)**. *Cnr2* expression was not modulated in PFC and NAc [***Cnr2***: (*PFC*: (F (2, 29) = 0.5527, *p* = 0.5813), (*NAc*: (F (2, 23) = 1.401, *p* = 0.2666)] but increased in DS [***Cnr2***: (*DS*: F (2, 28) = 4.192, *p* = 0.0256)] (**Figure 1B)**. *Cnrip* expression is not modified by the cocaine treatment in PFC and DS [***Cnrip***: (*PFC*: (F (2, 32) = 0.79, *p* = 0.4625), (*DS*: F (2, 30) = 2.172, *p* = 0.1315)] but decreased in NAc [***Cnrip***: (*NAc*: F (2, 28) = 5.84, *p* = 0.0076)] **(Figure 1B)**. We then performed a gene expression analysis of *Nape-Pld, Faah*, *Daglα* and *Mgll* in PFC, NAc and DS. We did not observed any changes of expression for any of the enzymes in any structures of interest **(Figure 1C)** [***Nape-Pld***: (*PFC*: (F (2, 32) = 0.8829, *p* = 0.4234), (*NAc*: F (2, 30) = 1.261, *p* = 0.2980), (*DS*: F (2, 32) = 0.5533, *p* = 0.5804)]; [***Faah***: (*PFC*: (F (2, 31) = 0.2618, *p* = 0.7714), (*NAc*: F (2, 29) =

0.02787, *p* = 0.9725), (*DS*: F (2, 29) = 0.1573, *p* = 0.8552)]; [***Daglα***: (*PFC*: (F (2, 31) = 0.5498, *p* = 0.5826), (*NAc*: F (2, 29) = 1.584, *p* = 0.2224), (*DS*: F (2, 25) = 1.432, *p* = 0.2576)]; [***Mgll***: (*PFC*: (F (2, 30) = 0.6629, *p* = 0.5227), (*NAc*: F (2, 31) = 0.01325, *p* = 0.9868), (*DS*: F (2, 31) = 0.6060, *p* = 0.5519)]. To explore whether these small changes could be due to either cocaine itself or passive cocaine intake we then used a cocaine-SA model that allows overcoming these aspects.

Transcriptional changes of cannabinoid receptors induced by cocaine self-administration

After 10 days of cocaine-self administration (FR1 at the dose of 0.33mg/kg), we performed a gene expression analysis 24h after last cocaine session in PFC, NAc, DS, HPC, Hab, Amy, LH, VTA and RMTg. First, cocaine injection number was stable across the 10 cocaine-SA sessions. Indeed, cocaine injection mean number was 89± 1.09 while the saline injection mean number for controls animals was 27± 1.91 **(Figure 2A)**. During the 10 sessions, the animals received a mean cocaine intake of 9.69 ± 0.11 mg/kg of cocaine. The difference between cocaine and saline injections was highly significant across all sessions [F (10, 70) =3, 7778, p=, 00042] **(Figure 2B)**. The difference between pressing the active versus the inactive lever reached significance on day 3 to last session [F (10, 70) =10,591, p=, 00000].

We found that CB1R and CB2R gene expression were both considerably altered compared to results obtained with intraperitoneal injections. *Cnr1* expression was increased by cocaine self-administration in NAc, DS and markedly increased in HPC [***Cnr1:*** *NAc*: *p* = 0.0019; *DS*: *p* = 0.0071; *HPC*: *p* = 0.0001] **(Figure 2C)**. On the opposite, *Cnr2* expression was two times decreased in PFC, DS and LH [***Cnr2:*** *PFC*: *p* = 0.0035; *DS*: *p* = 0.0010; *LH*: *p* = 0.0060] **(Figure 2C) (supplemental figure 1)**. In brief, *Cnr1* expression is mostly increased while *Cnr2* expression is on the opposite mostly decreased. These modifications indicate a potential cocaine-induced opposite regulation of CB1R and CB2R gene expression in most of brain reward-related structures.

In parallel to cannabinoid receptors gene expression analysis, we investigated gene expression changes of endocannabinoid enzymes for both synthesis and degradation. Concerning AEA enzymes, we observed that NAPE-PLD gene expression, enzyme for synthesis, was increased in NAc (*NAc*: *p* = 0.0066) **(Figure 2D)**. Gene expression of its enzyme for degradation, FAAH, was increased in PFC and strongly increased in HPC while its expression was decreased in NAc (*PFC*: *p* =0.0047; *HPC*: *p* = 0.0000; *NAc*: *p* = 0.0408) **(Figure 2D)**. Concerning 2-AG enzymes, we note a strong increase of its enzyme for synthesis gene expression, DAGLα, in the HPC (*HPC*: *p* = 0.0000) **(Figure 2D)**. These results suggest a cocaine-induced regulation of eCBs levels in PFC, NAc and HPC. Only few ECS enzymes alterations were observed in Hab, Amy, LH, VTA and RMTg **(supplemental figure 1)**.

Endocannabinoid levels modulated by cocaine self-administration

Considering the strong cocaine-induced regulations on enzymes gene expression, we further explore eCBs regulation levels. We measured AEA and 2-AG levels by mass spectrometry in PFC, NAc, DS and HPC. We first compare our measures with the ones described in the scientific literature. We found that our levels were similar to measurements already described (Buczynski & Parsons, 2010) **(supplemental figure 2)**. AEA levels were decreased in NAc and DS while its levels were increased in HPC in parallel to 2-AG and unaffected in PFC (*PFC*: $p = 0.1916$; *NAc*: $p = 0.0487$; *DS*: $p = 0.0280$; *HPC*: $p = 0.0201$) **(Figure 3A)**. We observed an increase of 2-AG levels in PFC, NAc and HPC (*PFC*: $p = 0.0137$; *NAc*: $p = 0.0025$; *HPC*: $p = 0.0334$) **(Figure 2D)**. 2AG levels did not change in DS (*DS*: $p = 0.9302$). Interestingly, we note an overall increase of 2-AG levels while AEA levels decrease in whole striatum and increase in HPC.

CB1 receptor expression and functionality modulated by cocaine self-administration

Previous transcriptional modifications results suggest a modulation of cannabinoids receptors. We assessed CB1R expression by western blot. We observed an increase of CB1R expression in both PFC and DS without any alterations in HPC (*PFC*: $p = 0.027$; *DS*: $p = 0.489$*; HPC*: $p = 0.1133$). We explored further CB1R protein expression density in subregions of structures of interest. CB1R expression was too low in other structures of interest such as the VTA and RMTg to be precisely measured. We were unable to measure CB2R expression due to technical difficulties. CB1R expression density was in agreement with the descriptive literature (Rivera *et al.*, 2014) **(Figure 3B, C)**. CB1R density expression was greater in DLS compared to controls (*DS*: $p = 0.0037$) **(Figure 3D)**. CB1R expression density remained unaffected in both NAc sub regions, nor in DMS and whole dorsal HPC (*NAcCo*: $p = 0.3104$; *NAcSh*: $p = 0.1973$*; DMS*: $p = 0.4938$; *HPC*: $p = 0.1070$) **(Figure 3D)**. CB1R density was then assessed in HPC sub regions CA1, CA2 and DG. We found no regulations of CB1R expression density (*CA1*: $p = 0.1311$; *CA3*: $p = 0.9196$*; DG*: $p = 0.4439$) **(Figure 3E)**.

To further characterize CB1R alterations, we investigated CB1R functional activity by GTPγS binding assay in PFC, DS and HPC. We used the specific CB1R agonist ACEA (ref GTP ACEA?) to test if cocaine modulates CB1R functional activity. The Emax was increased in HPC from cocaine treated animals compared to controls (*HPC*: $p = 0.0233$). We noted an insignificant small shift to the left for the EC50 in cocaine animals HPC (*HPC*: $p = 0.1169$). We did not find any changes in terms of Emax or EC50 in PFC and DS (*PFC*: $p = 0.1658$; *DS*: $p = 0.9912$*)* **(Figure 4)**.

Histone modifications involved in ECS transcriptional regulations

We next investigated whether the ECS transcriptional regulations in HPC involved histone modifications. We focused on H3K27Ac and H3K4Me3 marks as cocaine modulates these marks

(ref). We measured H3K4Me3 binding in the promotor regions of *Cnr1*, *Faah* and *Daglα* genes as their expression was strongly regulated in HPC. While promotor region of *Cnr1* was not affected by H3K4Me3 (*Cnr1*: *p* = 0.6375) **(Figure 5)**, we found an increased binding of H3K4Me3 in *Faah* and *Daglα* genes (*Faah*: *p* = 0.0189; *Daglα: p* = 0.0385) **(Figure 5)**. Concerning H3K27Ac, we found that *Faah* promotor and exon 1 region presented an increase binding of this mark *(Faah-prom*: *p* = 0.0383; *Faah-exon1*: p = 0.0066) **(Figure 5)** while *Cnr1* and *Daglα* were not affected *(Cnr1-prom*: *p* = 0.2295; *Cnr1-exon1*: p = 0.1764; *Daglα-prom*: *p* = 0.6454) **(Figure 5)**.

Chromosome conformation capture at CB1 promoter locus

To investigate further CB1R transcriptional regulations, we hypothesized that cocaine-SA might induce chromatin alterations at *Cnr1* gene locus. Indeed, using the 3D genome browser (http://promoter.bx.psu.edu), this chromatin interaction prediction tool suggest chromosomal loops of the *Cnr1* gene in mouse cortex. Therefore, we targeted *Cnr1* (Chr5) promoter region in HPC and NAc. Most interacting fragments were located on *Cnr1* chr5. In HPC, we obtained 2485561 and 19306922 reads in control and cocaine-SA group respectively whereas, in NAc, we measured 1372646 and 6824379 reads in control and cocaine-SA respectively **(supplemental figure 3)**. We then generated *Cnr1* 4C profiles from both regions and we noticed that overlay of 4C profiles are highly similar, suggesting that Cnr1 interactions are not structure dependent **(Figure 5)**. Chromatin looping can be altered by enhancers (Meng & Bartholomew, 2018) and as enhancers are associated to the H3K27Ac histone mark, we overlaid a H3K27Ac profile from rat hypothalamus (Toro *et al.*, 2018). We noticed the H3K27Ac profile fits nicely both 4C profiles, which may indicate that *Cnr1* interactions are associated to H3K27Ac. The analysis of both profiles revealed 14 and 2 significant fragments in NAc and HPC respectively. Even if we detected more interacting fragments changes in NAc compare to HPC chromatin changes were quite close to NAc chromatin alterations **(Figure 5)**. These data reveal that *Cnr1* expression is probably dependent of chromatin alteration. Even if H3K27Ac mark seems to be involved in these adaptations, our own data suggest that other mechanisms are involved in these alterations. Further studies should address whether these chromatin modifications are associated with either histone marks or DNA methylation. To further explain CB1R gene expression modulation, we used a transcription factor binding site prediction tool (https://zlab.bu.edu), transcription factors matrices were obtained from TRANSFAC (http://gene-regulation.com/). Cocaine regulates transcription factors such as SP1 (Imam *et al.*, 2003; Meyer *et al.*, 2009), Zif268 (Bhat *et al.*, 1992b; Hope *et al.*, 1992; Moratalla *et al.*, 1992; Daunais & McGinty, 1995; Valjent *et al.*, 2006; Besson *et al.*, 2013) and NFκB (Muriach *et al.*, 2010; Orso *et al.*, 2017).Therefore, we looked at their predicted binding site at CB1R gene locus and proximal regions to observe if their binding fits with chromatin alteration **(Figure 5)**. We detected only few Zif268 predicted binding sites and Nfkb predicted binding site have poor probability of binding. Interestingly, most of SP1 predicted

binding sites fit with chromatin alterations and have a better binding probability **(Figure 5)**. Thus, *Cnr1* expression may be associated with SP1 binding.

Discussion

Here, we showed that the ECS undergoes strong transcriptional changes induced by cocaine-SA in many brain reward-related regions compare to only few modifications triggered by cocaine intraperitoneal injections. Histone marks H3K4Me3 and H3K27Ac and chromatin conformation are associated with ECS gene expression in HPC and NAc. Overall, cocaine-SA increases ECS activity in HPC.

Voluntary versus passive intake

Both protocols clearly indicate that only cocaine voluntary intake strongly regulate the ECS at the transcriptional level. Models using intraperitoneal injections such as cocaine sensitization paradigms or chronic/repeated injections do not necessarily reflect cocaine-induced neuroadaptations. Therefore, our data strongly suggest that the ECS plays a role in voluntary cocaine intake. Moreover, our transcriptional data do not correlate with previous studies using cocaine sensitization in PFC and HPC (Blanco *et al.*, 2014, 2016). The only similar adaptation is CB1R gene expression increase in HPC (Blanco *et al.*, 2016). Therefore, our results suggest a better understanding of the cocaine induced ECS adaptations and its involvement in voluntary cocaine intake but as CB1R is expressed in different cell types (GABAergic, glutamatergic neurons and astrocytes), we are unable to specify in which cells these adaptations appear which limit our results interpretation.

CB2 gene expression

CB2R expression is far more complex to analyze as its expression level is very low in the central nervous system. CB2R is expressed in microglial cells (Van Sickle *et al.*, 2005b) and only few studies report its expression in postsynaptic neurons membranes (Zhang *et al.*, 2014; Stempel *et al.*, 2016). Many evidences show an involvement of CB2R in modulating inflammatory responses (Turcotte *et al.*, 2016). Not only CB2R activation trough eCBs or specific agonist exerts anti-inflammatory effects (Zoppi *et al.*, 2014; Malek *et al.*, 2015; Tang *et al.*, 2016) but also CB2-KO mice show an exacerbated inflammatory phenotype (Turcotte *et al.*, 2016). There are still discrepancies regarding the relationships between cocaine use and neuroinflammation. Nevertheless, cocaine users present higher levels of interleukine-6 (a pro-inflammatory cytokine) (Fox, D'Sa, *et al.*, 2012; Ersche *et al.*, 2014; Levandowski *et al.*, 2016; Moreira *et al.*, 2016). Moreover, post-mortem brain of cocaine users show an increase in activated microglia (Little *et al.*, 2009). If future studies confirm cocaine-induced modulation of neuroinflammation, our data

may indicate an enhancement of the inflammatory responses as CB2R gene expression is decreased in several brain structures in our conditions. Unfortunately, we were unable to analyze further CB2R potential adaptations.

Endocannabinoid levels

At first, eCBs levels measurement were surprising as studies usually indicate only few cocaine induced eCBs changes in brain reward-related regions (González, Fernández-Ruiz, *et al.*, 2002; Caillé *et al.*, 2007; Orio *et al.*, 2009; Bystrowska *et al.*, 2014). Cocaine induced regulations of eCBs in NAc are still mixed as eCBs levels remain unchanged or decreased after cocaine-SA (Caillé *et al.*, 2007; Orio *et al.*, 2009). Interestingly, we also found a decrease of AEA levels in NAc (Orio *et al.*, 2009). We then reproduced a 2-AG levels decrease in DS but we observed an opposite regulation in HPC (Bystrowska *et al.*, 2014). We argue that differences observed are may due to the time point we used to measure eCBs levels. eCBs levels were measured 24h after last cocaine session to investigate cocaine-induced long-lasting changes and avoid the observation of acute effects. Other studies either measured eCBs 2h after last cocaine session or during cocaine-SA. Therefore, we suggest cocaine induces mainly long-lasting changes on the ECS as Bystrowska et al. (Bystrowska *et al.*, 2019) suggest.

CB1R alterations

Measurements of CB1R protein expression indicate an increase in both PFC and DS, more precisely in DLS, along with no changes in HPC or its subregions. Moreover, we found using agonist-stimulated [^{35}S]-GTPγS binding assay an increase of CB1R functional activity in HPC. Thus, this result highlights an enhancement of CB1R functionality without any protein expression alterations. Interestingly, CRIP1A gene expression was also increased in HPC in our conditions. As CRIP1A enhances CB1R signaling in HPC (Guggenhuber *et al.*, 2016), CRIP1A could be at the origin of CB1R increased functionality in our conditions.

CB1 – memory - learning

Several evidences identify the ECS as a mediator of learning. For instance, JWH-081, a CB1R agonist, impaired object recognition and spatial memory in a Y-maze task (Basavarajappa & Subbanna, 2014). Moreover, URB597, a FAAH inhibitor, not only elevated AEA level in HPC but also impaired object recognition and decreased LTP in HPC (Basavarajappa *et al.*, 2014). Recently, deletion and re-expression of CB1R in hippocampal GABAergic neurons respectively abolished and rescued mediated learning in a sensory preconditioning task (Busquets-Garcia *et al.*, 2018). Furthermore, CB1R activation elicited by AEA differentially modulates short-term memory depending on the stress level (Campolongo *et al.*, 2012, 2013; Santori *et al.*, 2019). Interestingly,

these results point out a role for AEA in learning while there is still no evidence indicating a role for 2-AG. Overall, as we observed an increase of both CB1R functionality and eCBs levels in HPC, these data could be an indicator of CB1R mediated learning (Busquets-Garcia *et al.*, 2018). Recently, a study found that CB1R alters the sensitivity of animals towards cocaine intake and participates to the associative learning processes (Martín-García *et al.*, 2016).

The HPC is an essential structure to form and maintain of cocaine-context associations (Grant *et al.*, 1996; Childress *et al.*, 1999; Kilts *et al.*, 2001; Wexler *et al.*, 2001). Dorsal HPC mainly controls context-induced reinstatement. Thus, tetrodotoxin or muscimol-induced inhibition of dorsal HPC decreases context-induced reinstatement of cocaine seeking, whereas cue-induced reinstatement remains unaffected (Fuchs *et al.*, 2005, 2007). On the other hand, baclofen/muscimol-induced inhibition of ventral HPC attenuates both context- or cue-induced reinstatement of cocaine (Rogers & See, 2007; Lasseter *et al.*, 2010), heroin (Bossert & Stern, 2014; Bossert *et al.*, 2016), or alcohol (Marchant *et al.*, 2016) seeking. More recently, chemogenetic inhibition of dorsal HPC inputs to lateral septum attenuated context-induced reinstatement of cocaine seeking (McGlinchey & Aston-Jones, 2018). Altogether, these findings highlight the dorsal HPC as a crucial structure for the formation of cocaine-context associated memories.

Interestingly, cocaine also modulates hippocampal long-term potentiation (LTP). After 15 days of cocaine-SA, withdrawal for either 3, 30 or 100 days modulates LTP. LTP in the CA1 region of HPC was increased, unchanged and decreased respectively at 3, 30 and 100 days compared to the control group (Thompson *et al.*, 2004). Using a tetanization protocol (100Hz) to induce LTP in HPC, LTP was still enhanced in rats who underwent 10 days of extinction after cocaine-SA (del Olmo *et al.*, 2006). These results suggest long lasting changes induced by cocaine and which could play a major role in cocaine addiction. In HPC, CB1R are mainly expressed at inhibitory terminals (Katona *et al.*, 1999, 2000) as recently confirmed in a study using CB1-KO mice and mice where CB1R was specifically deleted in glutamate and GABA neurons. CB1R expression was mainly GABAergic in HPC (Martín-García *et al.*, 2016). Wilson and Nicoll first demonstrated that CB1 activation produces the inhibition of GABA release in HPC (Wilson & Nicoll, 2001). eCBs also induce long-term depression of inhibition (iLTD). By reducing GABA release, this effect is associated with LTP at excitatory synapses (Chevaleyre & Castillo, 2003). For instance, eCBs facilitate LTP at Schaffer collateral to CA1 pyramidal cells (Chevaleyre & Castillo, 2004). Moreover, iLTD involves potentiation of excitatory postsynaptic potential (EPSP)-spike coupling (Chevaleyre & Castillo, 2003). In our conditions, as CB1R functional activity is increased in HPC, this could lead to a decrease in GABA release participating to the increase of hippocampal LTP. Thus, our result suggests that the ECS could be at the origin of increased LTP in HPC.

Astroglial CB1R signal has been demonstrated in HPC (Navarrete & Araque, 2008, 2010; Gómez-Gonzalo *et al.*, 2015), neocortex (Min & Nevian, 2012), DS (Ruiz-Calvo *et al.*, 2018), Amy (Martin-Fernandez *et al.*, 2017) as well as in cortical and hippocampal human brain tissue (Navarrete, Perea, *et al.*, 2013). Astroglial CB1R may play a major role in behavior as specific CB1R deletion in astrocytes abolished the impairment of spatial working memory and in vivo LTD induced by cannabinoids (Han *et al.*, 2012). Hippocampal astrocytes have been shown to express functional CB1R coupled to Gq/11 proteins that stimulate phospholipase C-mediated IP3 production and activation of IP3 receptors in the internal calcium stores (Navarrete & Araque, 2008) compared to CB1R-Gi/o coupled in other cells type. More recent work indicates that astroglial CB1R are necessary for long-term object recognition memory consolidation (Robin *et al.*, 2018). These findings suggest a general and crucial role of astroglial CB1R in synapses.

Epigenetic and chromatin alterations

Both histone marks H3K4Me3 and H3K27Ac were enriched at the promotor and exon 1 region of *Faah* gene. These enrichments could explain the strong upregulation of *Faah* in HPC. Significant chromatin alterations at CB1R gene locus occurred mainly in NAc as genome-wide approaches already showed (Nestler, 2014). We did not find any chromosomal loop with other genes in both structures nor histone modifications at *Cnr1* promoter or exon 1 loci. *Cnr1* could be under the influence of DNAm as several previous studies already suggest (Wang *et al.*, 2008; Hong *et al.*, 2015; Mancino *et al.*, 2015; D'Addario *et al.*, 2017; Almeida *et al.*, 2019). Further work is needed to investigate whether cocaine changes DNAm level in *Cnr1* region and its causality on *Cnr1* expression with guide RNAs to target DNMT activity (Engmann *et al.*, 2017). Interestingly, *Cnr1*expression seems likely to be modulated by SP1 transcription factor. Future ChIp experiments should confirm its involvement in the modulation of CB1R expression.

To conclude, our data reveal that mainly cocaine voluntary intake affects the ECS in concert with long-lasting changes. Cocaine-induced modifications of CB1R gene expression may is under control of the 3D structure of chromatin but most likely not due to histone alterations. ECS increased activity in HPC may be the reason why cocaine induced LTP is facilitated in CA1 region. Most importantly, the role of the ECS in these processes has never been explored. Therefore, GABA-CB1R in CA1 region could modulate these responses. We suggest further studies should investigate the involvement of hippocampal and astroglial CB1R in these processes.

References

The references are available at the end of the thesis manuscript.

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Author contributions

DN and KB designed the experiments and wrote the manuscript; DN, DF and PR performed the experiments, collected data and analyzed the results under the guidance of KB. PR consulted on the design of behavioral experiments and JZ discussed the molecular analysis.

Conflicts of Interest: None

Figure 1

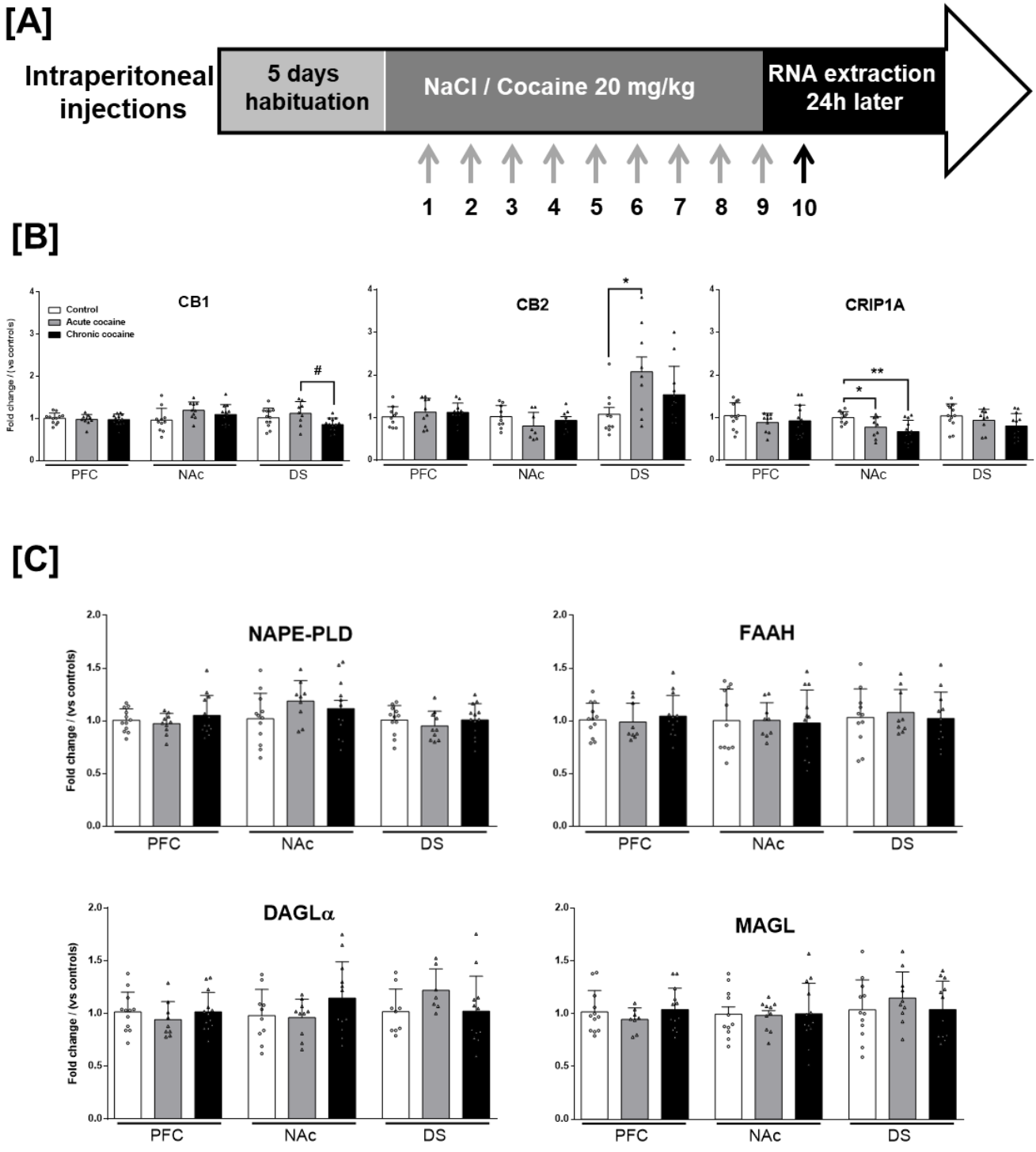


FIGURE 1: [A] Schematic representation of intraperitoneal cocaine administration(20mg/kg). Animals were injected once a day for 10 days with either saline (0,9%) or cocaine (20mg/kg). The dark arrow represents the acute injection on day 10. **[B]** Chronic cocaine injections decreases CB1 gene expression in DS compare to the acute cocaine treatment. CB2 gene expression is increased in DS by the acute cocaine treatment. CRIP1A gene expression is decreased by both cocaine treatments in NAc. **[C]]** ECS enzymes gene expression remained unchanged in PFC, NAc and DS, after both cocaine treatments. One-Way ANOVA + Newman–Keuls: *** $p<0.001$, ** $p<0,01$, *$p<0.05$ versus control group; # $p<0,05$ versus acute cocaine group

Figure 2

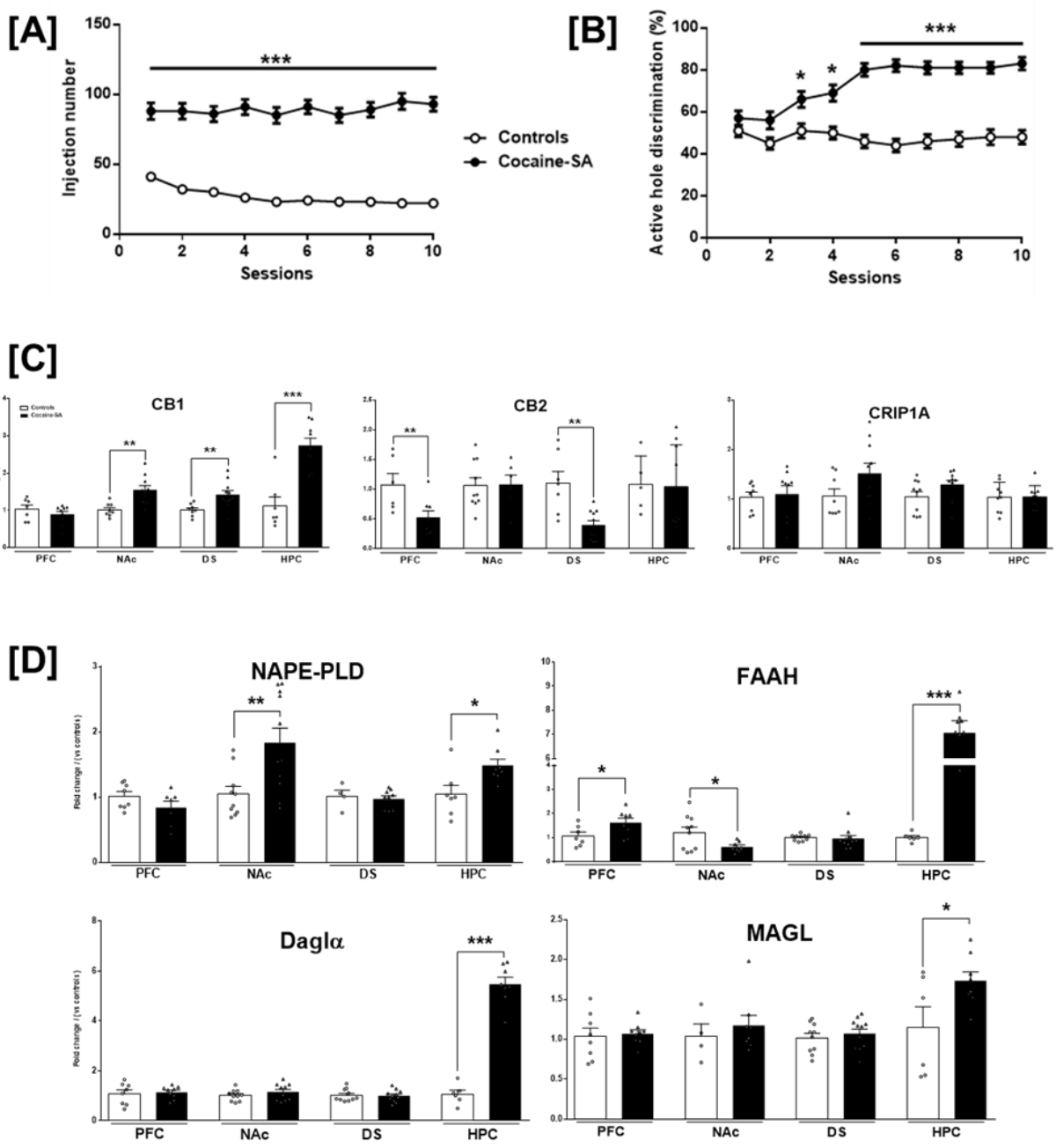


FIGURE 2: [A] Injection number per session. **[B]** Percentage of discrimination of the active hole. ANOVA repeated measures + Newman–Keuls: ***$P < 0.001$, *$P < 0.05$. Curves bars represent the mean ± SEM (n = 35-36/group). **[C]** Cocaine-SA increases CB1 gene expression NAc, DS and HPC. CB2 gene expression is decreased PFC and DS **[D]** Cocaine-SA induces an increase of Nape-PLD and FAAH gene expression in NAc and HPC. DAGLα gene expression is strongly increase in HPC following cocaine-SA. Student t-test: ***$P < 0.001$, **P< 0.01, *$P < 0.05$. Bars represent the mean ± SEM

Figure 3

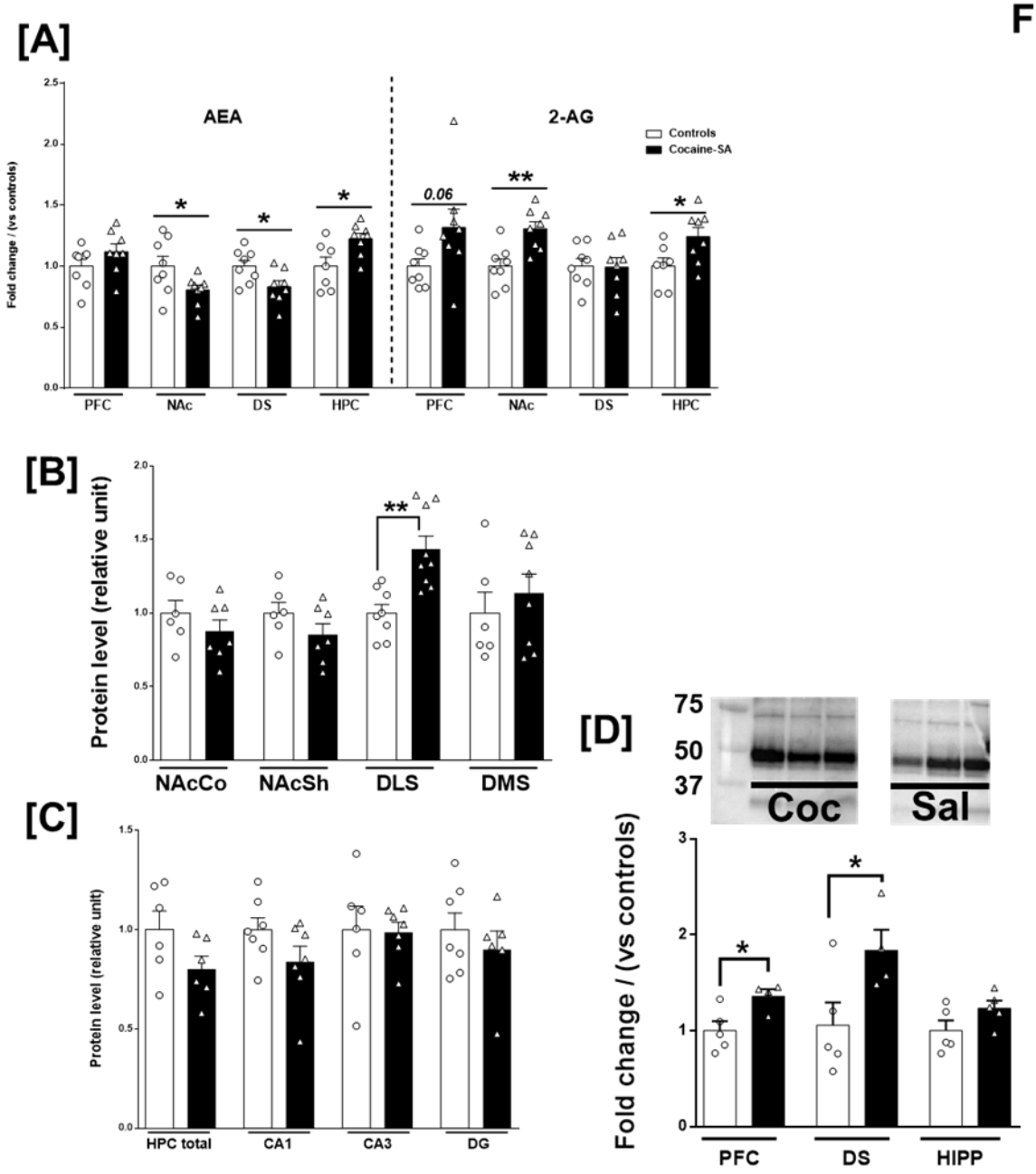


FIGURE 3: Cocaine-SA is associated with a modification of AEA and 2-AG levels and CB1R expression changes. **[A]** Endocannabinoid levels in PFC, NAc, DS and HPC measured by mass spectrometry. AEA levels are increased in the HPC and decreased in the DS and NAc while 2-AG levels are increased in the PFC, NAc and HPC. **[B]** Cocaine-SA increased its expression in the DLS. **[C]** CB1R expression remained unchanged in HPC and its sub-regions. **[D]** Cocaine-SA increased CB1R expression in the PFC and DS in western blot analysis.
Student t-test: *p<0.05 ; ** p<0,01. Bars represent the mean+S.E.M.

Figure 4

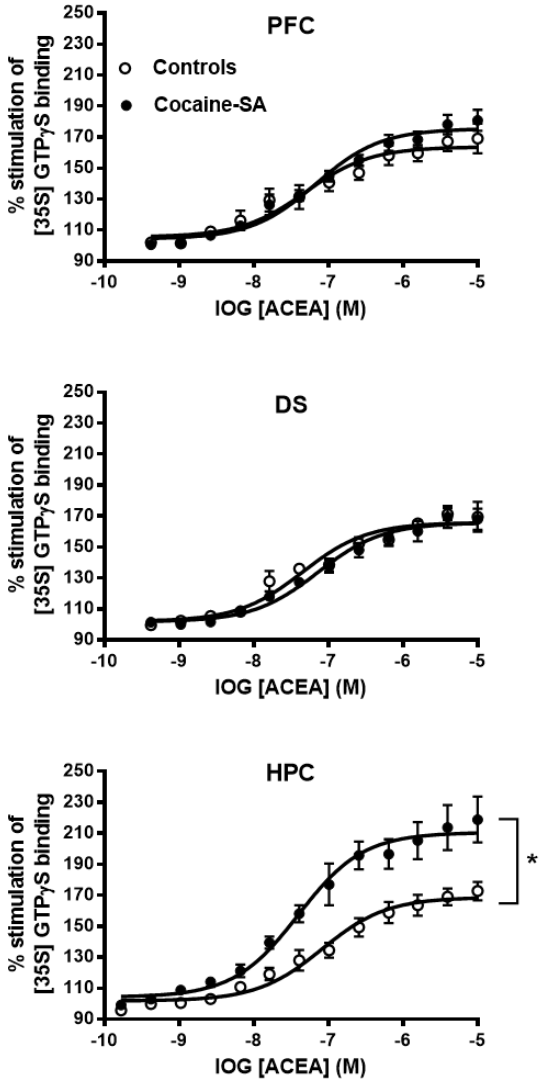


FIGURE 4: Cocaine-SA is associated with CB1R activity modulation. Curves represent CB1 activation in PFC, DS and HPC (Saline, n=4-7 and cocaine, n=7-8). CB1 activation is increased in the HPC and remain unchanged in the PFC and DS. Student t-test: $*p<0.05$.

Figure 5

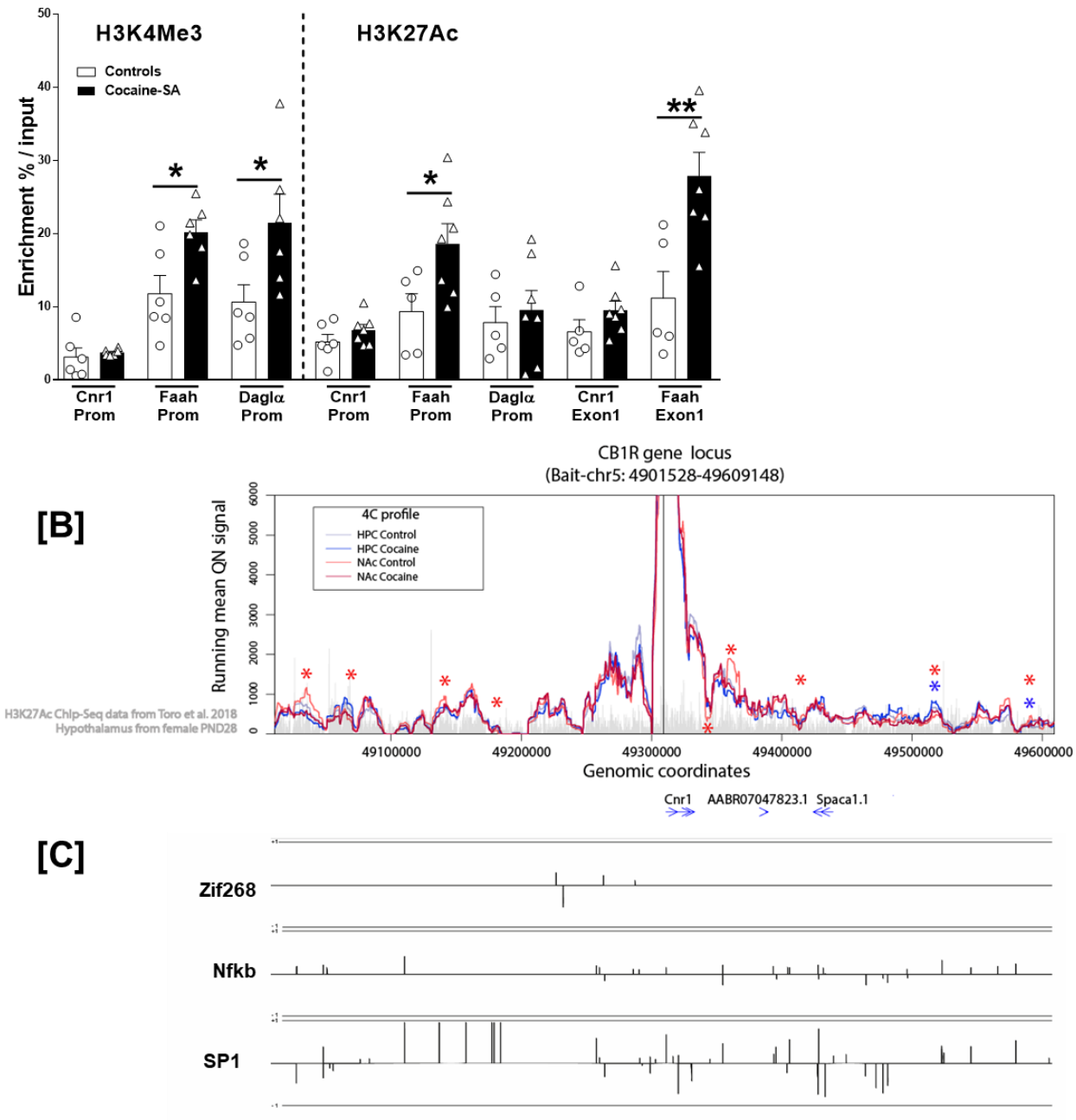


FIGURE 5: [A] Cocaine self-administration is associated with histones regulations on ECS associated genes. H3K4Me3 modifications in the HPC is associated with increased binding of H3K4Me3 on promotor regions of *Faah* and *Daglα* genes. H3K27Ac modifications in the HIPP with increased binding of H3K27Ac on promotor and Exon 1 regions of *Faah* gene. **[B]** Representative map of the 4C profile of the chromasomal interactions between CB1 gene and surrounding. Cocaine-SA induced several changes at the interaction level along CB1 gene region and surrounding **[C]** Representative map of transcription factors Zif268, Nfkb and SP1 binding site along CB1 gene region and surrounding. Student t-test : **p*<0.05 ; ** *p*<0,01. Histograms represent the mean+S.E.M.

Supplemental table 1: Primers

Target	Primer sens	Primer antisens
Rplp0	CTGCCCGAGCCGGTGCCATC	TTCAATGGTACCTCTGGAG
Cnr1	TCTGCTTGCGATCATCATGGTGT	AGATGATGGGGTTCACGGTC
Cnr2	AATGGCGGCTTGGATTCAA	TAGAGCACAGCCACGTTCTC
Nape-Pld	AGAGATCCGTGGCGATTCAC	ATCGTGACTCTCCGTGCTTC
Daglα	GGCATGGTACTCTCAGCTGA	GAGGAAGGAGAGAATGGCGG
Faah	CCCCAGAGGCTGTGTTCTTT	GTCAGATAGGAGGTCACGCA
MgII	GTTGAAGAGGCTGGACATGC	TCACGTGCTGCAACAAATCT
Cnrip	TTCCCGCATCTCTCTTGTCT	GTCCCGTTTACCGCTGTTTA
Cnr1-prom	AGAGGAACAGAATGCGTGCA	ATGAAGCCTCTTTCCCAGCC
Cnr1-exon1	TCATTTTAGGTCGTTAGGAGAACT	ACATACCTCAGCCATGGGTG
Faah-prom	GTGTTTTTGAGGGTGCCCAC	GTAGCTCCAACGGTCAAGGT
Faah-exon1	GAGCTGTTGGTTTGTGCGAG	CTAGGCAAACCCCGGAGAC
Daglα-prom	GCGGAGTGGGGTTTACGTAA	CTCCCCAAGGCCCCTATTCA
Cnr1 (4C)	GTGCCCGCGGCGATC	TCAAAAGACGTTTCTTGGTAAGATT TACAAGTCAT

Supplemental table 2

HPLC gradient

Time (min)	0	2.5	12.5	14.5	15	19
% B mobile phase	55	55	99	99	55	55

Supplemental table 3

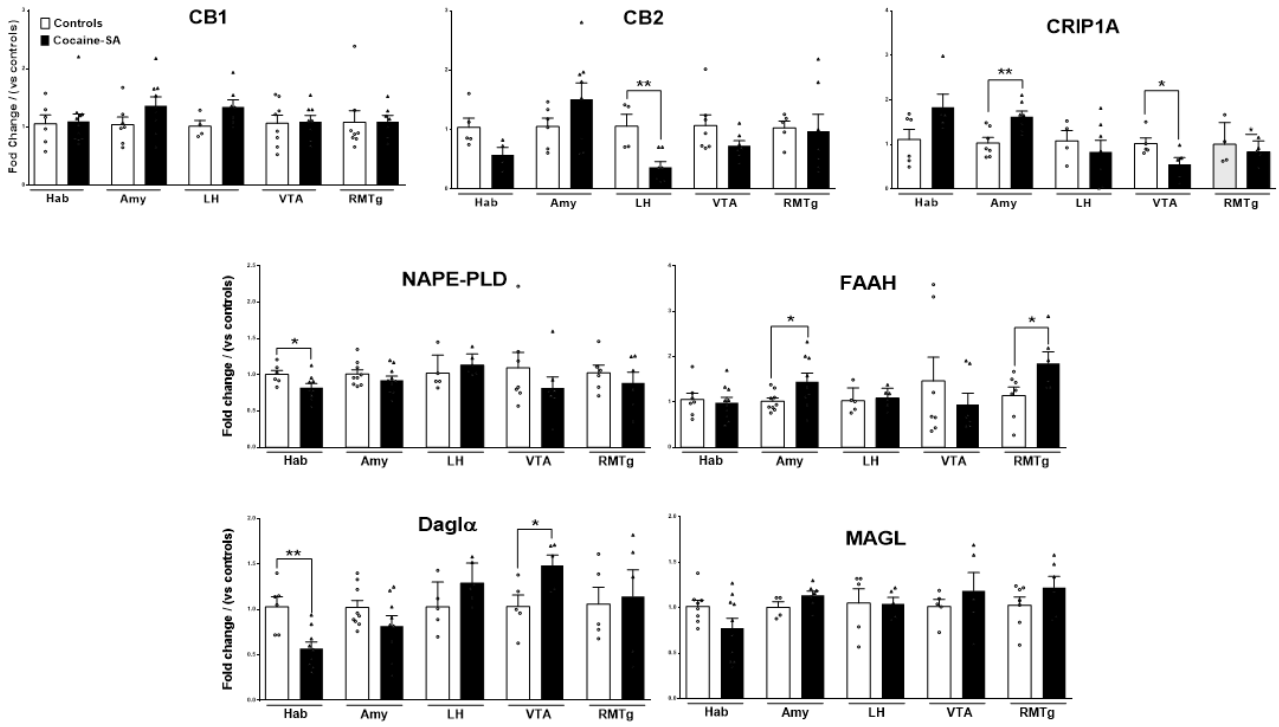
MS ionization, selection, fragmentation and identification parameters

Compound	Polarity	Precursor (m/z)	Product (m/z)	Ion product type	Collision Energy (V)	RF Lens (V)
2-AG	Positive	379.28	269.15	Qualification	16.6	164
			287.15	Qualification	14.6	
			361.22	Quantification	14.6	
D8-2-AG	Positive	373.28	209.17	Qualification	26.5	178
			294.33	Qualification	43.2	
			331.04	Quantification	39.0	
AEA	Positive	348.27	203.17	Qualification	17.0	207
			269.17	Qualification	17.1	
			287.15	Quantification	14.8	
D5-AEA	Positive	352.27	203.17	Qualification	17.4	208
			269.17	Qualification	17.1	
			287.15	Quantification	14.9	

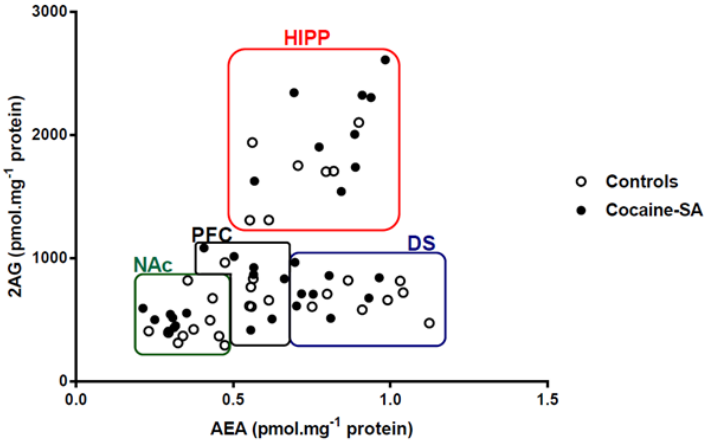
Supplemental table 4

Emax and EC50

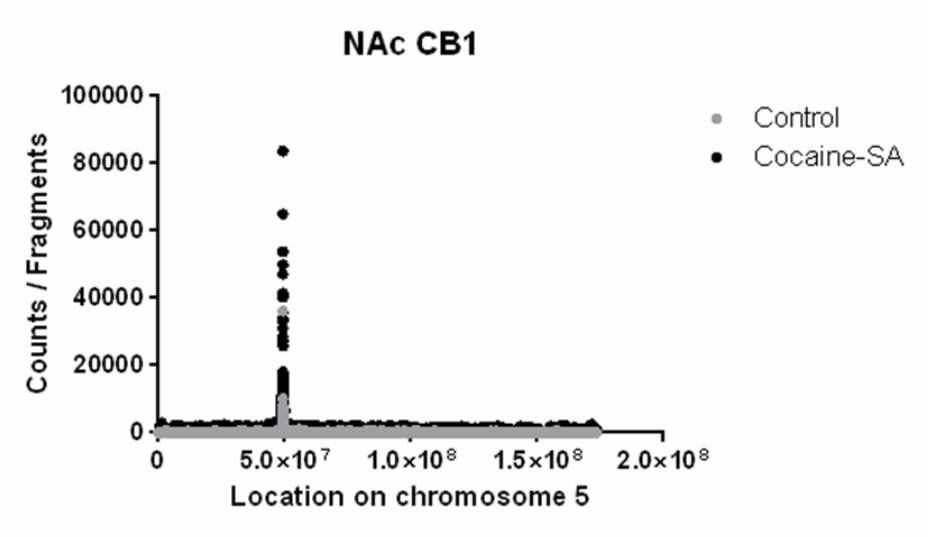
	Prefrontal Cortex		Dorsal Striatum		Hippocampus	
	Emax (%)	EC50 (nM)	Emax (%)	EC50 (nM)	Emax (%)	EC50 (nM)
Saline (n=6/8)	165,4±4,6	71,5	166,6±4,3	20,5	170,2±10,5	152,4
Cocaine-SA (n=9)	177,4±5,4	95,5	166,5±6,8	63,5	202,5±14,1	39,4

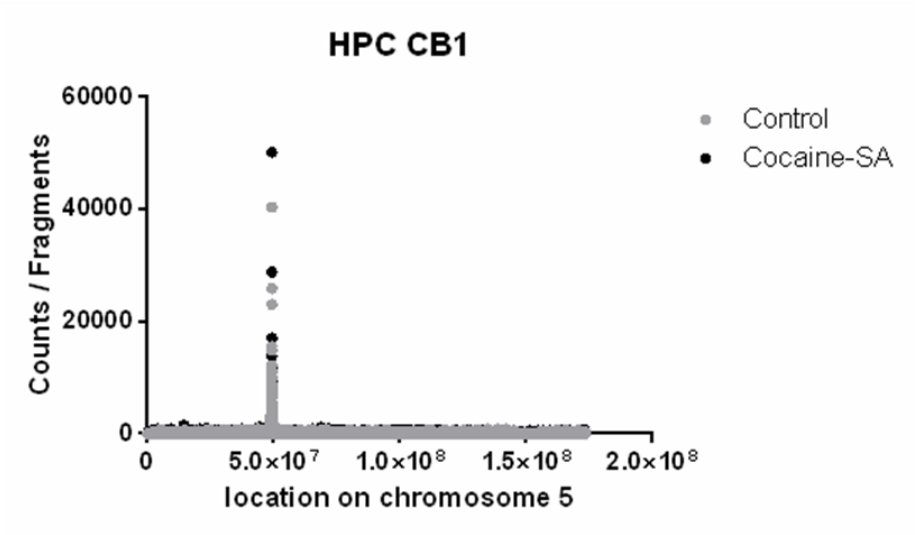


Supplemental FIGURE 1: ECS elements gene expression in Hab, Amy, LH, VTA and RMTg. CB2 gene expression is decreased IN LH. CRIP1A gene expression is increased and decreased in Amy and VTA, respectively
Student t-test: **P< 0.01, *P < 0.05. Bars represent the mean ± SEM
Nape-PLD gene expression is decreased in Hab. FAAH gene expression is increased in Amy and Rmtg. DAGLα gene expression is decreased in Hab and increased in VTA following cocaine-SA. Student t-test: ***P < 0.001, **P< 0.01, *P < 0.05. Bars represent the mean ± SEM

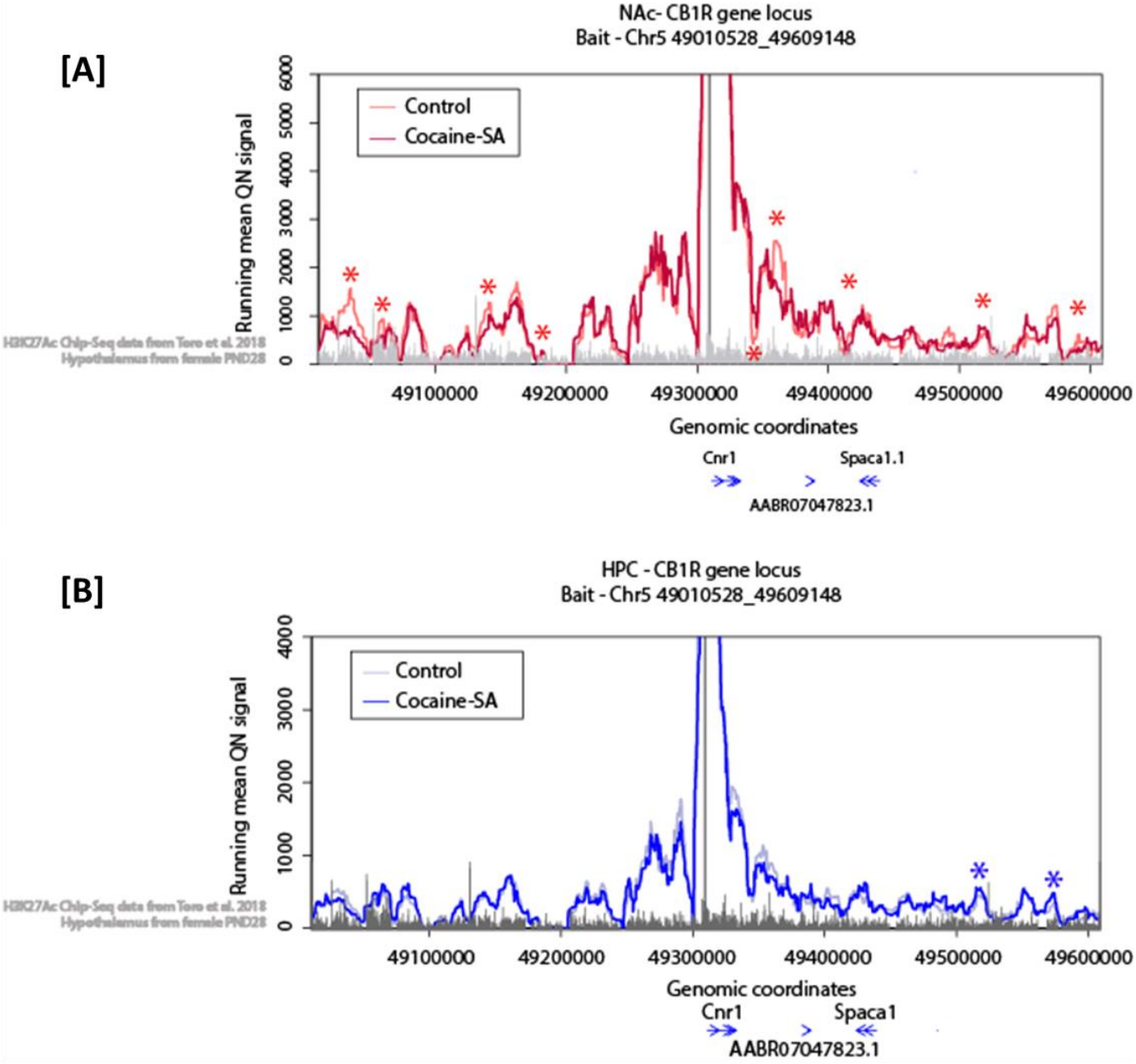


Supplemental FIGURE 2: Comparison endocannabinoid content levels in PFC, NAc, DS, and HPC. Our data is similar with the literature. For review see Buczynski et al. 2010





Supplemental FIGURE 3:



Supplemental FIGURE 4:

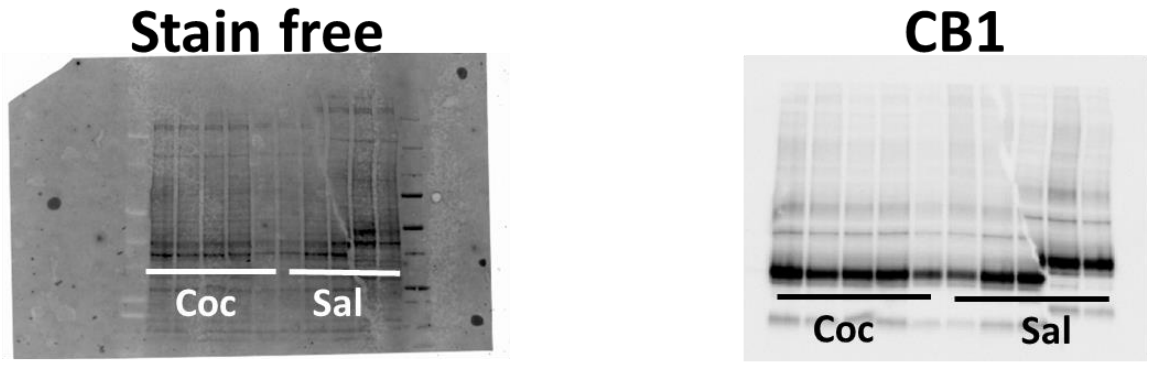
PFC


Stain free
Sal
Coc
CB1
Sal
Coc

DS


Stain free
Sal
Coc
Sal
CB1
Sal
Coc
Sal

HPC


Stain free
Coc
Sal
CB1
Coc
Sal

II. Article 2: voluntary cocaine intake modulates mu opioid receptors in the hippocampus

The opioid system is composed of three types of G protein-coupled receptors (GPCR) : mu opioid (MOP), delta opioid (DOP) and kappa opioid (KOP) receptors (Charbogne *et al.*, 2014; Befort, 2015; Bodnar, 2018; Darcq & Kieffer, 2018), respectively encoded by *Oprm1*, *Oprd1* and *Oprk1*. Also, three endogenous peptide families have been identified: β-endorphin, enkephalins, and dynorphins (encoded by *Pomc*, *Penk* and *Pdyn*) (Terenius, 2000), which preferentially bind to MOP, DOP, and KOP, respectively.

The OS is well known for its ability to modulate pain, drug reward and motivation (Kelley, 2004; Berridge & Kringelbach, 2008; Le Merrer *et al.*, 2009; Feng *et al.*, 2012). In the following paper, the introduction details that the OS can modulate cocaine intake and discuss that cocaine-induced alterations of the OS is rather inconsistent in the literature, except for the increase of Pdyn and Penk mRNA in whole striatum. I propose a table that recapitulates the main findings for this latter point, where most of studies focused on NAc and DS **(Table 7**). Also, only two studies before our investigation analyzed the effect of a voluntary cocaine intake in rodents (Sharpe *et al.*, 2000; Valenza *et al.*, 2016).

This study was performed in parallel to our main project examining cocaine-SA impact on the ECS in reward-related brain areas. Here, we investigated cocaine-induced OS alterations at the mRNA, epigenetic and functional levels, in our voluntary model of cocaine intake. We point to specific regulation in the HPC. The manuscript has been submitted to *Neuropharmacology* on 09/30/2019.

	NAc	DS	Amy	HPC	VTA	Administration	Publication
Acute cocaine	↑ Pdyn, Penk	↑ Pdyn, Penk				40mg/kg i.p	*(Helton et al., 1993)*
		↑ Pdyn				30mg/kg i.p	*(Svensson & Hurd, 1998)*
	↓ KOP				↓ KOP	Ethanol 2g/kg i.p + Cocaine 15mg/kg i.p	*(Rosin et al., 1999)*
	↓ KOP					45mg/kg i.p	*(Rosin et al., 2000)*
		↑ Pdyn				15mg/kg i.p	*(Yuferov et al., 2001)*
		↑ Pdyn				15mg/kg i.p	*(Zhou et al., 2002)*
				↑ Pdyn		20mg/kg i.p	*(Turchan et al., 2002)*
	↑MOP	↑MOP				15mg/kg i.p	*(Schroeder et al., 2003)*
		↑ Pdyn				30mg/kg i.p	*(Adams et al., 2003)*
	NC	NC	NC			15mg/kg i.p	*(Rosin et al., 2003)*

	NAc	DS	Amy	HPC	VTA	Administration	Publication
Chronic cocaine	↑MOP	↑MOP	↑MOP			10 or 30mg/kg i.p	*(Unterwald et al., 1992)*
	↑DOP	↑DOP				30 or 45mg/kg i.p	*(Unterwald et al., 1993)*
	↑MOP, KOP	↑MOP, KOP	↑MOP		↑KOP	45mg/kg i.p	*(Unterwald et al., 1994)*
		↑ Pdyn				30mg/kg i.p	*(Svensson & Hurd, 1998)*
		↑MOP				1mg/kg i.v + withdrawal	*(Sharpe et al., 2000)*
	↑MOP, KOP	↑MOP, KOP				30mg/kg i.p	*(Unterwald et al., 2001)*
		↑KOP				50mg/kg s.c	*(Collins et al., 2002)*
				↑Pdyn		20mg/kg i.p	*(Turchan et al., 2002)*
	↑MOP	↑MOP				15mg/kg i.p	*(Schroeder et al., 2003)*
	↓DOP	↓DOP				15mg/kg i.p	(Perrine *et al.*, 2008a)
	↑DOP					15mg/kg i.p	*(Ambrose-Lanci et al., 2008)*
	↑Pdyn	↑Pdyn				50mg/kg s.c	*(Caputi et al., 2014)*
	↑Pdyn ↓Pomc	↑Pdyn ↑Penk ↑MOP ↑DOP				0.2, 0.5, 1.25, 2.5mg/kg i.v	*(Valenza et al., 2016)*

Table 7: Cocaine-induced alteration of the OS in reward-related brain regions. *The PFC is not represented here as I found only one paper which found a decrease of DOP activity in this site* (Perrine *et al.*, 2008). *The lateral hypothalamus has also been investigated but no changes were detected in (Rosin et al., 2003). Legend: mRNA, protein, GTP, binding. NC: no changes.*

Voluntary cocaine intake modulates mu opioid receptors in the hippocampus

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Key words: mu opioid receptor, cocaine, hippocampus, gene expression, epigenetic

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Abbreviations:

Amy: amygdala ; Cocaine-SA: cocaine self-administration ; CPP: conditioned place preference ; DAMGO : [D-Ala2, N-MePhe4, Gly-ol]-enkephalin ; DOP: Delta opioid receptor; DS: dorsal striatum ; GPCR: G protein coupled receptor ; HPC: hippocampus; KOP: Kappa opioid receptor; LTP: long-term potentiation; MOP: Mu opioid receptor; OS: opioid system; Pdyn: Pro-dynorphin; Penk: Pro-enkephalin; PFC: prefrontal cortex; NAc: Nucleus Accumbens; VTA: ventral tegmental area.

Abstract

Cocaine addiction is a complex pathology induced by long term changes in the brain. Understanding the neurochemical changes underlying the reinforcing effects of drugs of abuse is critical for reducing the societal burden of drug addiction. The opioid system, consisting of endogenous opioid peptides and three opioid receptors mu, delta, and kappa, appears as a key player in reward. This system is modulated by chronic cocaine treatment in specific brain structures, but few studies investigated neurochemical adaptations induced by voluntary cocaine intake. In this study, we investigated whether intravenous cocaine-self administration (0.33mg/kg/injection, FR1, 10 days) in rats induces long term adaptations, including transcriptional and functional changes of components of the opioid system in reward related brain regions. In addition, epigenetic processes with histone modifications were examined for two activating marks, H3K4Me3 and H3K27Ac. We found an increase in Mu opioid receptor gene expression along with a potentiation of its functionality in hippocampus of cocaine self-administering animals compared to saline controls. We also observed marked modifications of Delta and Kappa receptors gene expression in nucleus accumbens and amygdala. Chromatin immunoprecipitation followed by qPCR revealed no modifications of the histone mark H3K4Me3 and H3K27Ac levels at Mu opioid receptor promoter. Our results show that voluntary intravenous cocaine self-administration in rats induces modifications of the opioid system in a region-specific manner with key effects in the hippocampus. Our study highlights the hippocampus as an important target to further investigate neuroadaptive processes leading to cocaine addiction.

Highlights

- Cocaine self-administration alters opioid transcripts in brain reward structures
- Cocaine self-administration increases Mu opioid receptor activity in hippocampus
- H3K4Me3 & H3K27Ac marks at Mu promoter region are not modulated by cocaine

Introduction

Addiction is a multi-factorial disease, involving genetic, neurobiological, psychological and environmental factors, that has detrimental consequences for individuals and society. According to the DSM-V, it is characterized by excessive intake through time, craving, compulsive drug use despite negative consequences and relapse (American Psychiatric Association, 2013; Koob & Volkow, 2016). Neuroplastic alterations are strongly implicated in the progression of addiction (Robinson & Kolb, 1999b, 1999a; Ron & Jurd, 2005), whereby altered gene expression impacts neuronal function and behavior (Contet *et al.*, 2004; Przewlocki, 2004; Rhodes & Crabbe, 2005; Spanagel & Heilig, 2005; McClung & Nestler, 2008; Russo *et al.*, 2010). For instance, cocaine causes widespread gene expression changes especially in reward-related brain structures such as the prefrontal cortex (PFC) and nucleus accumbens (NAc) (Kalivas *et al.*, 2005; McClung & Nestler, 2008; Russo *et al.*, 2010). Modifications also occur throughout the neuronal circuits involved in reward (Volkow & Morales, 2015). Interestingly, transcriptional neuroadaptations occurring in addictive behaviors persist for a long time after discontinuation of drug use (Becker & Le Merrer, 2016).

The opioid system (OS) is well known for its ability to modulate drug reward and motivation (Kelley, 2004; Berridge & Kringelbach, 2008; Le Merrer *et al.*, 2009; Feng *et al.*, 2012). Exogenous and endogenous opioids mediate their effects via activation of three types of G protein-coupled receptors (GPCR): mu opioid (MOP), delta opioid (DOP) and kappa opioid (KOP) receptors (Charbogne *et al.*, 2014; Befort, 2015; Bodnar, 2018; Darcq & Kieffer, 2018), respectively encoded by *Oprm1*, *Oprd1* and *Oprk1*. Three endogenous peptide families have been identified: β-endorphin, enkephalins, and dynorphins (Terenius, 2000), which preferentially bind to MOP, DOP, and KOP, respectively. Opioid receptors and peptides are widely expressed throughout the central nervous system, especially in reward-related brain areas (Le Merrer *et al.*, 2009). In particular, MOP is primarily expressed in dorsal striatum (DS), septum, hypothalamus, NAc, hippocampus (HPC) and ventral tegmental area (VTA) (Le Merrer et al., 2009). DOP

expression is higher in NAc, DS, amygdala (Amy) and HPC (Pradhan *et al.*, 2011; Chu Sin Chung & Kieffer, 2013; Erbs *et al.*, 2015) while KOP receptors are expressed in PFC, NAc, Amy, HPC and VTA (Chartoff & Mavrikaki, 2015).

As mentioned previously, the OS is strongly involved the modulation of drug reward. Pharmacological manipulation of opioid receptors can affect cocaine behavioral effects. For example, an infusion of CTAP, a MOP receptor antagonist, attenuated the development of a cocaine-induced conditioned place preference (CPP) in rat when injected into the NAc core, DS or VTA, and abolished it when injected directly into the NAc shell (Soderman & Unterwald, 2008). An VTA infusion of the MOP receptor agonist, DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin enhances the reinforcing effects of cocaine in a rat self-administration paradigm (Corrigall *et al.*, 1999). DOP receptors are also involved in cocaine behavioral effects in rat, as blockade of DOP receptors using naltrindole 5'-isothiocyanate (5'-NTII), a selective DOP antagonist, decreased cocaine self-administration (cocaine-SA) when infused into the NAc, and produced the opposite effect when infused into the VTA (Ward & Roberts, 2007). Also, the prototypic KOP agonist, U50.488, decreased cocaine self-administration and seeking in both rats (Glick *et al.*, 1995; Heinsbroek *et al.*, 2018) and Rhesus monkeys (Mello & Negus, 1998). A study conducted with Rhesus monkey showed that two KOP agonists, decreased cocaine self-administration and produced daunting side effects (Negus *et al.*, 1997). Systemic blocking of the KOP receptor with nor-binaltorphimine also attenuated cocaine self-administration and reinstatement in rats (Kuzmin *et al.*, 1998; Polter *et al.*, 2014), while the same antagonist had no effect on cocaine seeking, in Rhesus monkeys (Hutsell *et al.*, 2016). These findings suggest complex KOP implication in cocaine behavioral responses. Interestingly, two rat studies using cebranopadol, a mixed agonist targeting the three opioid receptors and another GPCR, the nociceptin receptor, reported a decrease of cocaine intake and a blockade of cocaine reinstatement (De Guglielmo *et al.*, 2017; Shen *et al.*, 2017), suggesting more complex pharmacological interactions implicating the OS. Taken together, these data support the fact that the three opioid receptors are involved in cocaine behavioral responses.

Genetic manipulation of the component of the OS using knockout mice also revealed a role for this system in psychostimulant behavioral responses (for review, see (Charbogne *et al.*,

2014)). For example, cocaine reward is reduced in cocaine-SA (Mathon *et al.*, 2005) and cocaine-CPP (Hall *et al.*, 2004) paradigms in MOP knockout mice. Cue-induced relapse following cocaine-SA was significantly weaker in both MOP and DOP knockout mice than in wild-type animals, while Pdyn knockout mice showed a slower extinction and increased relapse (Gutiérrez-Cuesta *et al.*, 2014). Also, DOP and Penk deficient mice showed less motivation for cocaine (Gutiérrez-Cuesta *et al.*, 2014). Cocaine CPP was unchanged in both KOP and Pdyn deficient mice, and remained unmodified in stress situation in comparison to control animals which showed potentiated CPP, indicating that the kappa/dynorphin system contributes to the stress-mediated response (McLaughlin *et al.*, 2006). This was supported by another study reporting that stress-induced reinstatement of cocaine was abolished in Pdyn deficient mice (Redila & Chavkin, 2008). Also, cocaine CPP was absent in β-end deficient mice (Nguyen *et al.*, 2012). In summary, these genetic studies and others (for a review see (Charbogne *et al.*, 2014) indicate a strong involvement of both opioid receptors and peptides in cocaine-induced reward related behaviors.

Cocaine induces neuroadaptations of the OS in reward-related brain structures (for a review, see (Yoo *et al.*, 2012), but specific findings are controversial, depending on experimental conditions and the structures under study. In studies investigating the effect of passive cocaine treatment on opioid gene expression, chronic cocaine infusion in rat elevated *Oprm1* expression (Azaryan *et al.*, 1998) and both MOP binding and functionality (Izenwasser *et al.*, 1996) in NAc, as well as KOP receptor expression in NAc, DS, claustrum and endopiriform nucleus (Collins *et al.*, 2002). DOP receptor gene expression and functionality remained unaffected by cocaine in NAc and DS (Azaryan *et al.*, 1996; Izenwasser *et al.*, 1996). In contrast, a study comparing Fisher and Lewis rats revealed an increase of *Pdyn* and *Oprd1* expression in DS of both strains following cocaine-SA (Valenza *et al.*, 2016). Whether these distinct results on gene expression were due to voluntary versus passive cocaine intake was not clear. Indeed, effects of cocaine administration on gene expression alteration (Saad *et al.*, 2019) and on LTP in VTA (Chen *et al.*, 2008) differed depending on voluntary (cocaine-SA) versus non contingent (cocaine-yoked) or passive administration. On this basis, the first aim of our study was to identify potential changes in gene expression of components of the OS in voluntary cocaine intake, in several rat brain structures involved in reward. We further investigated alteration of MOP receptor functionality in PFC, DS

and HPC. Finally, as cocaine is often associated with both histone marks, trimethylated lysine 4 (H3K4Me3) and acetylated lysine 27 (H3K27Ac) of histone 3 (Nestler, 2014; De Sa Nogueira *et al.*, 2019), we examined whether MOP receptor gene regulation could be related to histone modifications at MOP receptor promotor level, in the HPC.

Materials and Methods

Subjects

71 male Wistar rats (Janvier, France) weighting 250–300 g at the beginning of the experiments were habituated for two weeks to housing conditions in a temperature and humidity-controlled environment with a reverse 12 h light/dark cycle (lights on at 7:00 PM). Rats were grouped housed 5 animals/cage in standard cages with *ad libitum* access to food and water until surgery and then single-housed. All experimental procedures were performed in agreement with the EU Directive 2010/63/EU for animal experiments and approved by the institutional ethics committee CREMEAS (Comité d'Éthique pour l'Expérimentation Animale de Strasbourg, France, APAFIS#2015012716049550). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drugs

Cocaine hydrochloride was obtained from Cooper (1164500, Melun, France) and dissolved in sterile NaCl 0.9%. DAMGO was obtained from Sigma Aldrich (E7384, St Louis, USA).

Surgery

Intravenous catheterization procedure was performed as previously described (Fonteneau *et al.*, 2017). Briefly, rats were anesthetized by i.p infusion (1 ml/kg) of a mixture containing 90 mg/kg of ketamine (Imalgene 1000®, Centravet, France) and 10mg/kg of xylazine (Rompun®, Centravet), prior to surgical implantation of a chronic indwelling catheter in the right jugular vein. The silicone catheter (Silastic®, Plastics One, Roanoke, VA, USA), was fitted to a 23-

gauge guide cannula that was bent at a right angle and then embedded in dental cement on a circular 2.5 cm mersilene mesh base. A discrete incision was performed onto the jugular vein then the heparinized catheter was immediately inserted 3.6 cm into the vein and anchored with suture. Catheters were flushed daily with 150 µl saline solution containing 100 U/ml heparin and 50 mg/ml ampicillin to prevent clotting and infection, respectively.

Cocaine self-administration procedure

Drug self-administration was performed as previously described (Romieu *et al.*, 2011; Fonteneau *et al.*, 2017) in dark operant chambers (30 × 30 × 30 cm) located in a sound-attenuated room. Briefly, a computer driven syringe pump (Imetronic, Pessac, France) activated a 10 ml syringe and pushed fluid into Silastic® tubing connected to the rat through its externalized 23-gauge guide cannula. Each chamber was equipped with two ~~3~~ 2.5cm-diameter holes on the same wall, located 4 cm above the floor. Disruption of an infrared photobeam in each hole (nose-poke) was detected using a digital input card (DIO-24; National Instrument, Austin, TX, USA) and homemade LabView software (National Instrument). Nose-pokes into the inactive hole had no programmed consequence. Nose-pokes into the active hole triggered the i.v. administration of a 0.33 mg/kg dose of cocaine hydrochloride (60 µl over 2 sec) under control of the computer. A 5-sec flashing light, located 8 cm above the active hole, was paired contingently with the delivery of cocaine~~.~~ A 40 s-time-out period began simultaneously to the cocaine infusion. A fixed-ratio 1 (FR1) cocaine self-administration paradigm was carried out for 10 days during daily 2h sessions. Number of self-injections and nose pokes in active and inactive holes were recorded. Animals were given an overdose of pentobarbital (100 mg/kg, i.v) 24hr after the last session followed by decapitation and brain extraction to collect tissues of interest. PFC, NAc, DS, HPC (dorsal part) and Amy were microdissected and samples were immediately frozen on dry ice and kept at -80°C.

Quantitative real-time PCR

Samples were processed to extract total RNA using Ribozol (VWR) according to manufacturer's instructions. RNA quality (260/280 ratio: 1.8–2) and quantity was measured with a NanoVue™ (GE healthcare) spectrophotometer. Total RNA (750 ng) was reverse transcribed

into cDNA in a 20 µl final volume, with iScript (iScript™ cDNA Synthesis Kit, Biorad, France). Real-time PCR was performed using a CFX96 Touch™ apparatus (Biorad, France) and Sso Advanced™ Universal SYBR Green supermix (Biorad, France) in a final volume of 15 µl. Thermal cycling parameters were 30 sec at 95°C followed by 40 amplification cycles of 5 sec at 95° and 45 sec at 60°C. Primer sequences for all tested genes are given in **Table 1**. Expression levels were normalized to *rplp0* housekeeping gene levels and compared between controls and cocaine-SA samples using the $2^{-\Delta\Delta}$Ct method (Livak & Schmittgen, 2001).

Agonist-stimulated [^{35}S]-GTPγS binding assay

The [^{35}S]-GTPγS binding assay was used to measure G protein activation following MOP receptor stimulation with DAMGO, as previously described (Scherrer *et al.*, 2004). Tissues were homogenized in sucrose 0.25M, then centrifuged at 1100g (4°C) for 10 min. Supernatants were collected and centrifuged at 30000g (4°C) for 30 min. Pellets were then homogenized in sucrose 0.32M and subjected to Bradford analysis for total protein concentration, and then stored at −80 °C. The assay was initiated by the addition of cell membranes (50 µg) to the assay buffer (50mM Tris-HCl pH 7.4; 3 mM MgCl2; 0.2mM EGTA; 100mM NaCl) containing 0.1nM [^{35}S]-GTPγS (NEG030H, PerkinElmer, Courtaboeuf, France), 30 µM GDP and DAMGO (10^{-9}M to 10^{-4}M) for 1 h at 25 °C. Radioactivity was detected using a Top-Count scintillation counter (PerkinElmer, Billerica, MA, USA). Basal [^{35}S]-GTPγS binding was determined in the absence of agonist, and non-specific binding by replacing [^{35}S]-GTPγS by 10 µM non-radiolabeled GTPγS. Stimulated specific binding was converted in percentage of basal specific binding, defined as 100%. Data were analyzed using Prism 6 GraphPad software. Four to seven independent assays were performed in triplicate on three distinct membrane preparations per group. Stimulation (%), EC50s and IC50s were calculated for each experiment and averaged.

Chromatin immunoprecipitation

Samples were prepared as previously described (Caputi *et al.*, 2014) with minor modifications. Frozen tissues were ground on carbonic ice before fixation for 8 min with 1% formaldehyde and then quenched with glycine (0.125M) for 5 min. Tissue fragments were washed

with cold phosphate-buffered saline supplemented with protease inhibitors (#4693132001, Roche, France). Tissues were then homogenized in 900µl of sonication buffer (0.5% SDS, 10mM EDTA, 50mM Tris-HCL, pH8) and lysates were sheered using a diogenode bioruptor XL at 4°C, at high sonication intensity for 30s ON / 30s OFF for 50 min, to obtain DNA fragments <600 bp. Next, samples were centrifuged at 20000g (4°C) for 20min. Protein A magnetic beads (Dynabeads, Invitrogen) were coated with the respective antibody of interest (H3K4Me3, ab8580; H3K27Ac, ab4729, Abcam, France) at 4°C overnight on a rotator. Following washing of the magnetic beads and associated immune complex, they were added to 200µl of sheared chromatin for histone ChIPs and incubated overnight at 4°C on a rotator. A sample of each sheared chromatin (20µl, 10%) was used as input controls. Samples were washed with RIPA buffer (HEPES-KOH 50mM pH7.5, LiCl 500mM, EDTA 1mM, NP-40 1%, Na-Deoxycolate 0.7%) and elution buffer (SDS 1%, $NaHCO_3$ 100mM) on the next day. Reverse cross-linking was performed at 65°C overnight, and proteins and RNA were removed with proteinase K and RNAse A (ThermoFisher, USA) respectively according to manufacturer instructions. DNA was purified using a DNA mini elute kit (Qiagen, France). Additionally, a negative control without IgG was performed to test for nonspecific binding. PCR of MOP genomic sequence around the promoter region associated with the immunoprecipitated proteins was performed, as described above. Primers are indicated in Table 1.

Statistical analysis

All results are expressed as mean ± S.E.M. Behavioral data were analyzed using a repeated-measures two-way ANOVA followed by post-hoc Newman-Keuls tests (GraphPad Software 6.0, San Diego, CA). Other data were analyzed using student t-test. Significance was set at $p \leq 0.05$.

Results

Acquisition of cocaine self-administration

Details regarding our paradigm of cocaine-SA are represented in **Figure 1A**. The number of cocaine infusion was stable across the 10 cocaine-SA sessions. Indeed, the mean number of cocaine infusions was 89± 1.09, while the mean number of saline infusions for control animals was 27± 1.91 **(Figure 1B)**. During the 10 sessions, animals received a mean cocaine dose of 32.65±2.18 mg/kg **(Figure 1B)**. The difference between cocaine and saline infusions was highly significant across all sessions [F (10, 70) =3, 7778, p=, 00042] **(Figure 1B)**. Rats receiving saline did not differentiate between holes (50% discrimination). The difference between groups for active versus inactive hole pokes reached significance from day *2* to the last session [F (10, 70) =10,591, p=, 00000] **(Figure 1B)**.

Transcriptional changes of opioid receptors induced by cocaine self-administration

We measured opioid-related gene expression in several brain-reward related structures, as changes in gene expression might indirectly indicate altered opioid tone in these brain regions. The analysis was performed 24h after the last cocaine session in PFC, NAc, DS, HPC and Amy, in order to explore mRNA expression in the absence of an acute effect of cocaine.

We found that MOP gene expression was significantly decreased by cocaine-SA in DS [*MOP: DS*: *p* =0.0295] and, in contrast, increased 1.85 fold in HPC [*MOP: HPC*: *p* = 0.0448] **(Figure 2A)**. No changes could be detected in the other regions. Regarding DOP gene expression, we noticed a significant mRNA expression in the PFC, NAc and HPC [*DOP: PFC*: *p* = 0.0401; *NAc*: *p* = 0.0011; *HPC*: *p* = 0.0077] and a decrease in the Amy [*DOP: Amy*: *p* = 0.0029] **(Figure 2A)**. No changes were observed in the DS. Concerning KOP gene expression, as for the DOP receptor, we observed a significant 2.38 fold increase in the NAc [*KOP: NAc*: *p* = 0.0001], and a decrease in Amy [*KOP: Amy*: *p* = 0.0396] **(Figure 2A)**, but no changes in the DS or HPC. These modifications indicate differential effects of cocaine on each opioid receptor gene expression, which are brain structure dependent.

Transcriptional changes of opioid peptides induced by cocaine self-administration

In parallel to expression of the opioid receptors, we investigated gene expression of the precursors *Pdyn* and *Penk,* other components of the OS, within the same brain structures. We observed a significant up-regulation of Pdyn gene expression in DS ([***Pdyn:*** *DS*: *p* = 0.0098] while no regulations occurred in any of the other structures investigated **(Figure 2B)**. Concerning Penk gene expression, we observed a significant mRNA increase in both NAc and HPC [***Penk:*** *NAc*: *p* = 0.0396; *HPC: p* = 0.0022] **(Figure 2B)**, while no changes were detected in the other brain regions.

Cocaine self-administration modulates MOP functional activity

To further characterize MOP receptor alterations, we investigated MOP functional activity by [^{35}S]-GTPγS binding assay in the PFC, DS and HPC. We used the specific MOP agonist DAMGO to test whether cocaine modulates MOP functional activity (efficacy and potency). Interestingly, a significant effect was observed in HPC, with an increased efficacy (Emax) in cocaine-SA rats compared to controls (*Emax:* Control = 145.8 ± 1.8; Cocaine-SA = 164 ± 2.9*; HPC*: *p* = 0.0149) **(Figure 3)**. The potency (EC50) was not altered in this region (*EC50:* Control = 1,465.10^{-7}M ± 0.11; Cocaine-SA = 1,863.10^{-7}M ± 0.12). No significant changes of either Emax or EC50 were observed in PFC and DS **(Figure 3)**.

Histone modifications at MOP promoter region

To further investigate MOP transcriptional changes in HPC, we examined epigenetic modifications at MOP promoter region in this structure. As cocaine is often associated with activating marks, we targeted both H3K4Me3 and H3K27Ac. Enrichment percentage compared to input in both conditions remained unchanged [*H3K4Me3: p* = 0.6482 *H3K27Ac: p* = 0.1263], indicating no significant change in histone modification in HPC **(Figure 4)**. DOP gene expression was markedly increased in our conditions in the HPC, but we were not able to measure histone modifications due to technical difficulties with the primers targeting its promotor region.

Discussion

Our results show that voluntary intake of cocaine induces transcriptional changes of components of the OS in several reward-related brain regions. Interestingly, we observed region-

specific differences in gene expression, and in particular, a marked increase of MOP/DOP/Penk in the HPC. Increase of MOP transcript was correlated with increased functionality of this receptor in the same brain structure. Levels of the epigenetic marks H3K4Me3 and H3K27Ac were not associated with MOP gene expression changes observed in HPC.

KOP/Pdyn transcript regulations

It is well described that the KOP/Pdyn system is affected by cocaine at the NAc level (Wee & Koob, 2010). In agreement with previous reports, our data revealed an increase of KOP mRNA expression in the NAc. Such a regulation was observed in mice receiving daily cocaine for 7 days (Eipper-Mains *et al.*, 2013). Increased KOP binding was detected in the NAc in post mortem studies in humans, who died of cocaine overdose (Staley *et al.*, 1997; Mash & Staley, 1999). In contrast, other studies using passive protocols have shown a decrease of KOP gene expression (Rosin *et al.*, 1999, 2000) in the NAc, indicating mixed results even among passive paradigms. Cocaine-SA paradigms in rodents, but not in primates, also trigger Pdyn gene up-regulation in the NAc (Hurd *et al.*, 1992; Daunais & McGinty, 1995; Fagergren *et al.*, 2003), a finding that we did not observe at the mRNA level. Other ambiguous data have been reported for regulations taking place in the DS. Several studies indicated a 20% increase of KOP receptor binding in DS following passive administration of cocaine (Unterwald *et al.*, 1994, 2001; Collins *et al.*, 2002; Yoo *et al.*, 2012). Interestingly, one study using escalating cocaine-SA intake reported an increase of Pdyn gene expression in two rat strains, while the increase of KOP receptor transcripts in DS was significant in Lewis (escalating cocaine consumption) but not in Fisher (stable cocaine consumption) rats (Valenza *et al.*, 2016). Our own data indicate an increase of Pdyn mRNA in DS while KOP gene expression remained unchanged. We also observed a significant decrease of KOP gene expression in the Amy, a brain structure involved in emotions, a result in agreement with a specific role of KOP/Pdyn system in mood processing (Lalanne *et al.*, 2014). Also, KOP activation in the Amy of individuals diagnosed with cocaine dependence has been associated with craving, stress and increased cocaine relapse risk (Xu *et al.*, 2013). The KOP/Pdyn system in Amy is also involved in responses to other drugs of abuse. This was recently shown using specific inhibition of Pdyn neurons with a designer receptor exclusively activated by designer drugs approach, which

reduced binge-like alcohol drinking in mice (Anderson, Lopez, *et al.*, 2019). Interestingly, KOP gene expression decrease in Amy could also reflect early withdrawal adaptations, as we examined the mRNA levels 24h post last session. Indeed, withdrawal from cocaine administration has been shown to induce a reduction of KOP binding in several structures, including the basolateral Amy in rodents (Turchan *et al.*, 1998; Bailey *et al.*, 2007). Overall, there is a large body of evidence from preclinical models indicating that rewarding properties of cocaine alter both KOP expression and binding (Wee & Koob, 2010; Charbogne *et al.*, 2014; Lalanne *et al.*, 2014). The KOP/Pdyn system forms a distinct system within the OS, and has been proposed as an anti-reward system (Goldstein *et al.*, 1979; Chavkin *et al.*, 1982). Its activation reduces dopamine release, thereby decreasing the reinforcing properties of drugs of abuse (Lalanne *et al.*, 2014). We do observe an increase of gene expression of this anti-reward system, however, an effect on behavior may be masked by the marked up-regulations of the MOP/DOP/Penk genes.

MOP/DOP/Penk transcript regulations

Knockout mice for either MOP or DOP receptors exhibited decreased cocaine-SA and cue-induced relapse (Mathon *et al.*, 2005; Gutiérrez-Cuesta *et al.*, 2014) and the absence of Penk expression induced a reduction of motivation for cocaine (Gutiérrez-Cuesta *et al.*, 2014), indicating a role for the MOP/DOP/Penk system in cocaine responses. Nevertheless, reports of regulation of MOP, DOP and Penk gene expression by cocaine have been rather inconsistent (unchanged, up- or down-regulated) in the literature, specifically depending on the paradigms used (for a review see (Yoo *et al.*, 2012) and references therein). Our data showed an increase of both DOP and Penk mRNA expression in the NAC, while MOP gene expression was unchanged. MOP up-regulations in the NAc were previously observed in passive paradigms (Unterwald *et al.*, 1992, 1994; Izenwasser *et al.*, 1996; Azaryan *et al.*, 1998; Eipper-Mains *et al.*, 2013) while no changes were observed in a cocaine-SA paradigm, similar to our findings (Valenza *et al.*, 2016).

In our conditions, we could only detect a decrease of MOP gene expression in the DS while both DOP and Penk were not regulated. This observation was not associated with a change in functional activity of the receptor, as shown by our GTPγS binding results. Our data regarding the DS is in contradiction with a previous work reporting increased gene expression of MOP following

escalating cocaine-SA (Valenza *et al.*, 2016). Also, upregulation were observed in DS at mRNA (Unterwald *et al.*, 1992, 1994, 2001) or functional level (Bailey *et al.*, 2007; Schroeder *et al.*, 2003) in passive chronic cocaine paradigms. MOP binding was also up regulated at early withdrawal stage in cocaine users (Zubieta *et al.*, 1996). Also, DOP signaling was attenuated in the DS after repeated cocaine exposure (Unterwald *et al.*, 1993; Perrine *et al.*, 2008). These discrepancies on MOP/DOP expression in DS may therefore arise from the various paradigms or from methodological technologies used for the analysis.

Gene expression of the MOP/DOP/Penk system was increased in the HPC in our paradigm. To our knowledge, gene expression of the OS has not been investigated earlier in this brain structure following cocaine-SA. Whether the observed gene up-regulation is specific to the cocaine-SA paradigm or would also be observed in passive cocaine intake is not known. Interestingly, enhancement of MOP gene expression was correlated with an increased activity in HPC, as revealed by our GTPγS study. This is similar to results reporting an increase of MOP receptor (binding or GTPγS studies) following heroin-SA and cannabinoid-SA (Fattore *et al.*, 2007) in HPC. Altogether, this may reflect a role for MOP in learning or memory processes associated with reward. Indeed, the HPC is a crucial structure for the formation and maintenance of cocaine-context associations (Grant *et al.*, 1996; Childress *et al.*, 1999; Kilts *et al.*, 2001; Wexler *et al.*, 2001). In particular, the dorsal HPC controls context-induced reinstatement. Its inhibition by tetrodotoxin or muscimol attenuated context-induced, but not cue-induced reinstatement of cocaine seeking (Fuchs *et al.*, 2005, 2007). On the other hand, ventral HPC inhibition with baclofen/muscimol diminished both context- and cue-induced reinstatement of cocaine (Rogers & See, 2007; Lasseter *et al.*, 2010), heroin (Bossert & Stern, 2014; Bossert *et al.*, 2016), or alcohol (Marchant *et al.*, 2016) seeking. More recently, a study showed that chemogenetic inhibition of dorsal, but not ventral hippocampal inputs to lateral septum specifically attenuated context-induced reinstatement of cocaine (McGlinchey & Aston-Jones, 2018). Among potential mechanisms involved, the metabotropic glutamate receptor 1 has been proposed as selective antagonist decreased context-induced reinstatement of cocaine seeking when injected directly into dorsal HPC (Xie *et al.*, 2010). These findings together with our data highlight a role of hippocampal MOP in cocaine addiction.

Cocaine-SA induced long term potentiation (LTP) in the HPC has been reported after short withdrawal in CA1 region (Thompson *et al.*, 2004). Interestingly, MOP activation is partly responsible for mossy fiber LTP in hippocampal CA3 (Derrick & Martinez, 1994) and mice deficient for MOP exhibited an impairment of spatial learning, that may be associated with LTP deficit observed in mossy fibers in CA3 of these mice (Jamot *et al.*, 2003). Interestingly, recent data indicate MOP expression in CA1 astrocytes and demonstrate astroglial MOP-mediated LTP in HPC (Nam *et al.*, 2018, 2019). Future studies should investigate whether astroglial MOP are sufficient to modulate cocaine induced LTP in HPC. Our data indicate that DOP and Penk mRNAs expression also increase in HPC in our conditions. DOP activation or Penk release facilitate LTP in HPC (Chavkin *et al.*, 1985; Bramham *et al.*, 1991; Bramham & Sarvey, 1996). Moreover, a study revealed an increase of DOP gene expression in HPC in a spatial discrimination task (Robles *et al.*, 2003) and mice deficient for DOP were impaired in a drug-associated cue learning task, suggesting a hippocampal dysfunction (Pellissier *et al.*, 2016). Altogether, even though little information is yet available, the current data suggest that MOP/DOP/Penk system facilitates learning process associated to the context in cocaine-SA.

Also, we can hypothesis that cocaine induced transcriptional changes of the MOP/DOP/Penk system may have crucial consequences at the protein expression level in specific brain structures. Indeed, a recent finding have highlighted that following chronic morphine administration, DOP receptor expression was decreased in CA1, CA3 and DG, and this decrease persisted after 4 weeks of abstinence (Erbs *et al.*, 2016). Interestingly, this study also highlighted that MOP/DOP neuronal co-expression was wider in morphine dependent mice and this co expression was detected in novel brain areas located in circuits related to drug reward and emotional processing underlying withdrawal (Pierre *et al.*, 2019). Similar adaptations of MOP/DOP may occur following cocaine-SA intake and it would be of interest to investigate functional interactions across receptors (MOP/DOP heteromer or opioid receptor/another receptor) under such drug treatment.

Epigenetic adaptations

It is well described that cocaine can induce epigenetic changes in specific brain structures (Nestler, 2014; De Sa Nogueira *et al.*, 2019). This can occur through regulation of epigenetic factors involved in DNA methylation (MeCP2) or histone modifications (HDACs) (Zwiller, 2015) or through direct modifications at histone level. To investigate whether increased expression of MOP gene in HPC was under the control of such modifications, like H3K4me3 and H3K27ac, we analyzed these activating marks at the level of MOP gene promotor. Our data indicate that these activating marks do not participate to MOP gene regulation observed in the HPC, as no specific changes of H3K4me3 and H3K27ac levels were found in our conditions. Previous work has shown a regulation of H3K4Me3 mark following cocaine intake in the HPC of postmortem brains of cocaine abusers (Zhou *et al.*, 2011) and another study proposed this mark as an OS element regulator in striatum (Caputi *et al.*, 2014). Histone modifications were connected to increased Pdyn gene expression in NAc (decrease of a repressive mark H3K27me3) and in the lateral DS (increase of an activating mark, H3K4me3) (Caputi *et al.*, 2014). A decrease of Pdyn gene expression in the medial part of DS was connected to a reduction of the H3K4me3 activating mark (Caputi *et al.*, 2014). These data illustrate that the OS can be under the control of epigenetic regulations.

Among other epigenetic changes at OS system, DNA methylation has been described at MOP promotor region (Hwang *et al.*, 2007, 2010; Nielsen *et al.*, 2009, 2010; Chorbov *et al.*, 2011) but no report concerning cocaine exposure is yet available. Exposure to opiate in human revealed an increased DNA methylation at several CpG sites in the MOP promoter region (Nielsen *et al.*, 2009; Doehring *et al.*, 2013). In humans with alcohol and nicotine codependence, a study identified several methylated CpG among which three occurred respectively in DOP, KOP and Penk genes (Xu *et al.*, 2017). Altogether, this suggest that DNA methylation could be involved following cocaine use at the level of OS gene that we will further examine.

Conclusions

Overall, our data highlighted up-regulation of MOP/DOP/Penk gene expression in the HPC following voluntary cocaine intake. Further examination of the role of the OS in the HPC and associated epigenetic changes, will be crucial for the development of therapeutic treatments.

References

The references are available at the end of the thesis manuscript.

Acknowledgements

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Author contributions

DN and KB designed the experiments and wrote the manuscript; DN, DF and PR performed the experiments, collected data and analyzed the results under the guidance of KB.

Conflicts of Interest: None

Table 1

Target	Forward primer	Reverse primer
Rplp0	CTGCCCGAGCCGGTGCCATC	TTCAATGGTACCTCTGGAG
MOP	ACAGCCTGTGCCCTCAGACC	CAGGAAGTTTCCGAAGAGGCC
DOP	GCTCGTCATGTTTGGAATCG	AGGTACTTGGCGCTCTGGAA
KOP	ACTGGCATCATCTGTTGGTA	GGAAACTGCAAGGAGCATT
Penk	AGCCAGGACTGCGCTAAAT	AGGCAGCTGCCCTTCACATT
Pdyn	GAGGGCAGTGCCTTTCCCAAG	TTAATGAGGGCTGTGGGAA
MOP promoter region	GGCCAGAGATGCATCGATCA	GAGTCAGCCTGGCCAAGATT

Legends to figures

Figure 1

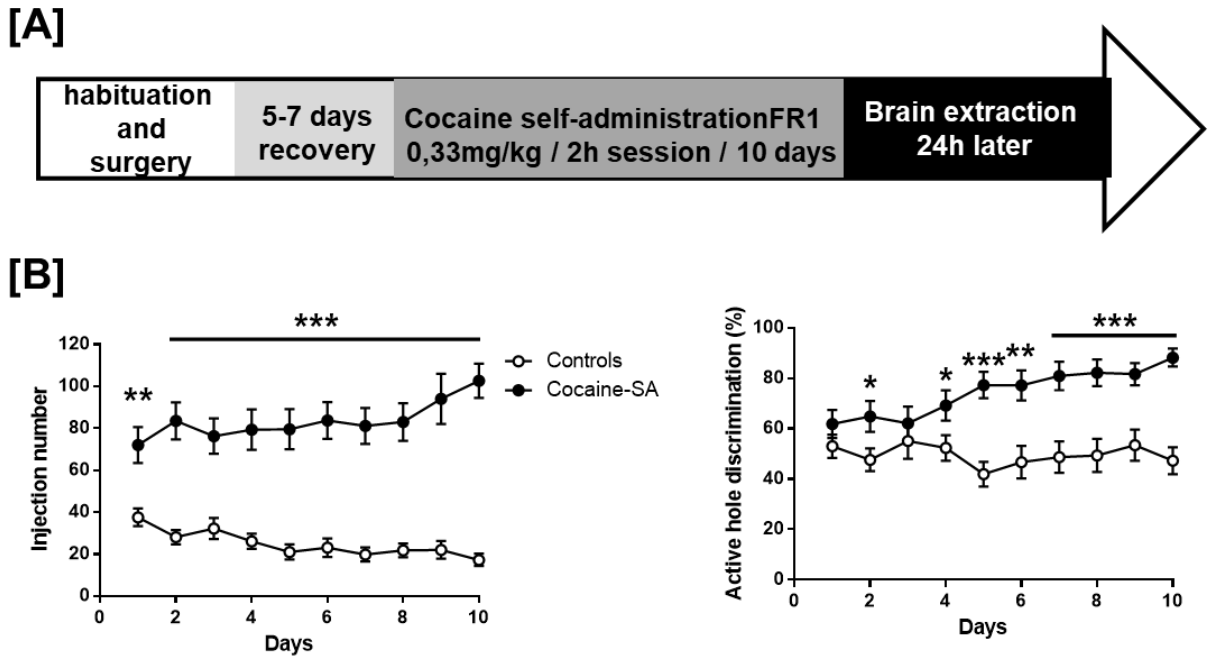

FIGURE 1: Cocaine self-administration in rats. [A] Schematic representation of the cocaine-SA paradigm. Rats underwent surgery for self-administration and following a week of recovery, had access to the operant chamber 2h per day, for 10 days. Each cocaine infusion delivered 0.33mg/kg of cocaine. **[B]** The number of cocaine injections per session and the percentage of discrimination of the active hole versus the inactive hole are presented. ANOVA repeated measures + Newman–Keuls: ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. Curves represent the mean ± SEM (n = 35-36/group).

Figure 2

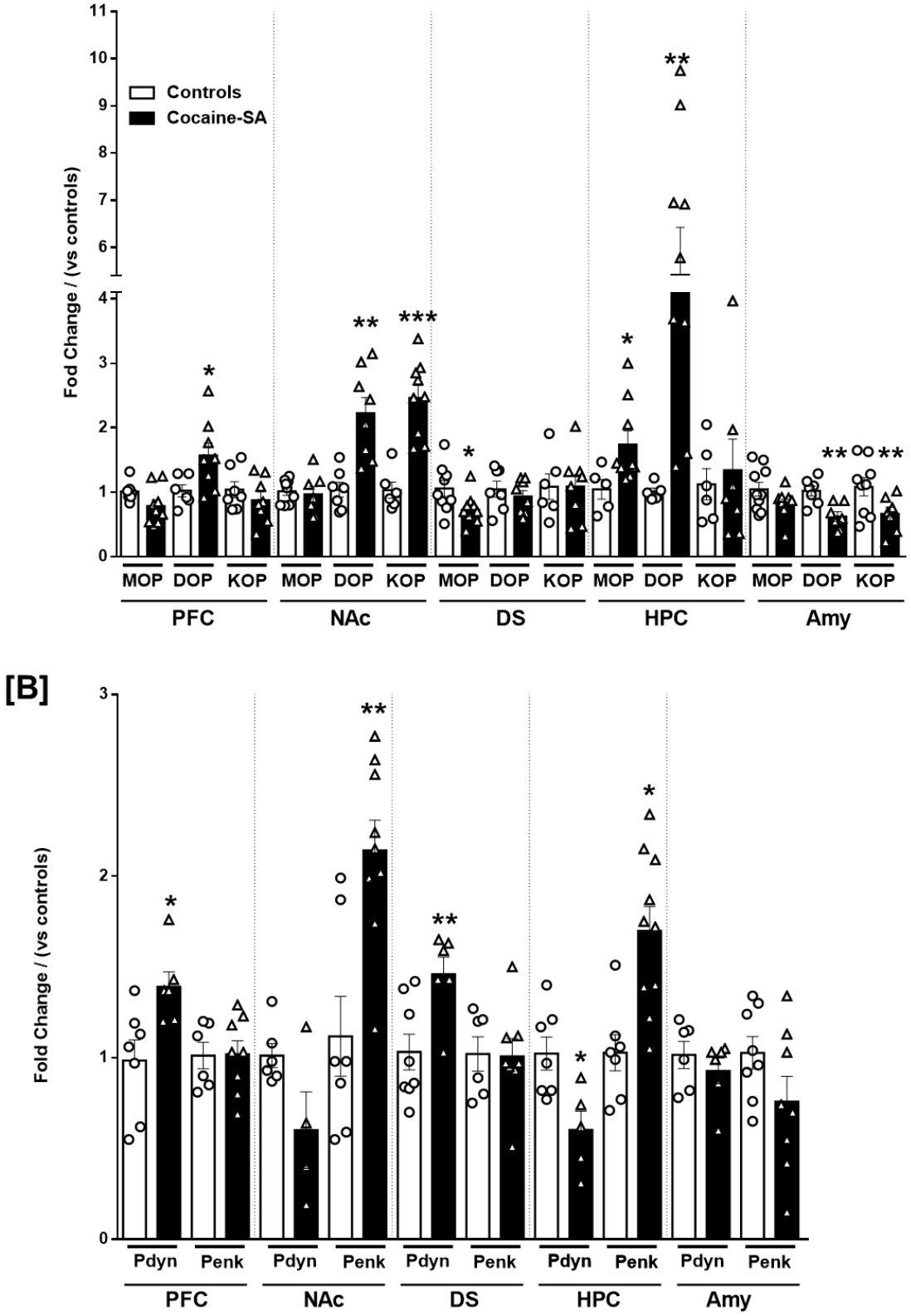
[A]
Controls
Cocaine-SA
Fod Change / (vs controls)
MOP DOP KOP
PFC
NAc
DS
HPC
Amy
[B]
Fold Change / (vs controls)
Pdyn
Penk
PFC
NAc
DS
HPC
Amy

FIGURE2: Cocaine-SA regulates OS gene expression in reward-related brain regions. [A] OS receptor gene expression was altered by cocaine-SA. MOP gene expression is increased in the HPC and decreased in the DS. DOP gene expression is strongly increased in PFC, NAc and HPC and decreased in AMY. KOP gene expression is increased in the NAc and decreased in the Amy. **[B]** Pdyn and Penk gene expression were regulated by cocaine-SA. Pdyn gene expression is increased in PFC, DS and decreased in HPC. Penk gene expression is increased in NAc and HPC. Student t-test: *** p<0.001, ** p<0,01, *p<0.05. Histograms represent the mean + S.E.M n= 4-10/group.

Figure 3

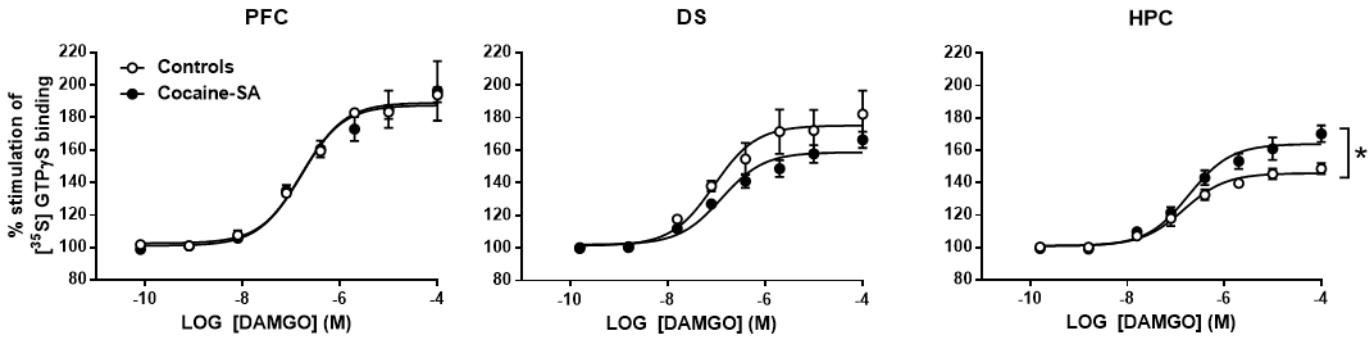

FIGURE 3: Cocaine-SA increases MOP receptor activity. Curves represent MOP activation by DAMGO, a MOP receptor agonist, in PFC, DS and HPC (Saline, n=4-6 and cocaine, n=4-7) as measured by [^{35}S] GTPγS binding. MOP activation was increased in HPC and remained unchanged in PFC and DS. Mean + S.E.M.

Figure 4

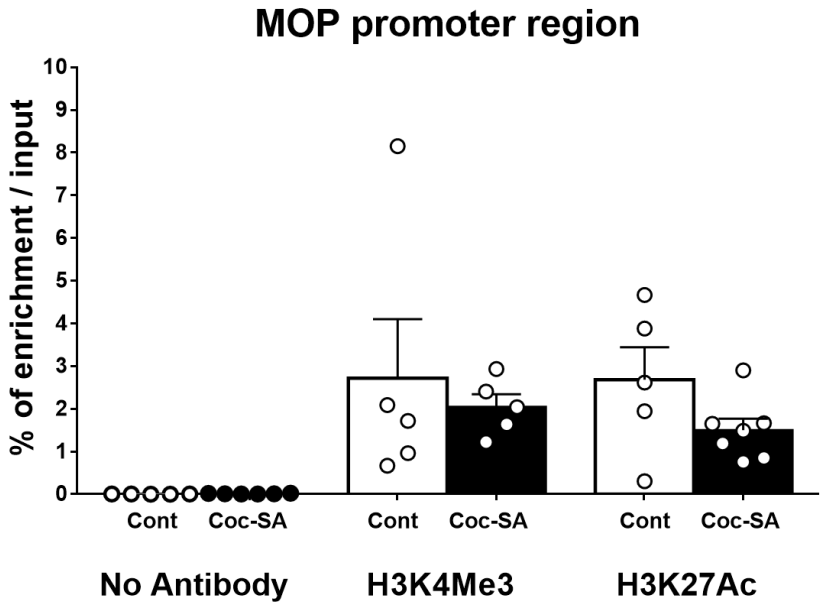

FIGURE 4: MOP promoter histone modifications following cocaine-SA. No differences in terms of enrichment of histone marks were found at MOP promoter region for both H3K4Me3 and H3K27Ac between cocaine-SA animals and controls. Enrichment was specific to both antibodies as we found no enrichment without antibody. Histograms represent the mean + S.E.M. n= 5-7/group. Cont = controls; Coc-SA = cocaine-SA.

III. Article 3: transcriptomic analysis of binge sucrose-induced neuroadaptations: a focus on the endocannabinoid system

1. Interactions between the ECS and food

As mentioned previously, Cannabis is very well known for its ability to stimulate appetite (Abel, 1975). From the 30's to 70's, several studies confirmed the stimulating effects of Cannabis on appetite but they mainly focused on acute effects (see review (Cota *et al.*, 2003)). Then studies relying on chronic treatment demonstrated that weight gain, food palatability (sweet or fat food) and the social aspect of smoking were associated with food intake increase in both humans and rodents (Foltin *et al.*, 1988; Mattes *et al.*, 1994; Williams & Kirkham, 1999; Koch, 2001). In particular, Cannabis stimulates appetite for sweet foods (Mattes *et al.*, 1994). Noteworthy, Yoshida et al. demonstrated a cannabinoid-induced increase of gustatory nerve responses to sweeteners without affecting responses to salty, sour, bitter, and umami compounds in mice (Yoshida *et al.*, 2010). This effect was absent in CB1-KO mice (Yoshida *et al.*, 2010) suggesting a crucial role of CB1 in sugar intake. Indeed, studies demonstrated that Rimonabant reduced sweet food intake (Simiand *et al.*, 1998) associated with an attenuation of dopamine release evoked by presentation of palatable food (Melis *et al.*, 2007). Moreover, Rimonabant dose-dependently decreased both food intake and body weight (Colombo *et al.*, 1998). This compound was therefore proposed as a medication for weight loss, however, Rimonabant has been removed from market following severe side effects such as depressive behaviors and suicidal thoughts in patients. To date, targeting only peripheral CB1 receptors has been suggested as a better strategy for treating obese patients (Argueta & DiPatrizio, 2017).

Recent studies employed operant procedures with palatable food. For instance, in adolescent rats trained to self-administer chocolate-flavored pellets, motivation for palatable food was higher compared to elder rats (Méndez-Díaz *et al.*, 2019). Interestingly, CB1 and CB2 expression was higher in elder rats compare to adolescents in PFC, NAc and HPC which may be at the origin of the behavioral differences in terms of food seeking (Méndez-Díaz *et al.*, 2019). In mice self-administering corn oil, rimonabant decreased responding during extinction and reinstatement (Ward *et al.*, 2009). Moreover, a CB2 antagonist slightly decreased food-SA (sweetened milk) (Adamczyk, Miszkiel, *et al.*, 2012) while both JWH133 and CBD attenuated sucrose-SA in rodents (Zhang *et al.*, 2015; Bi *et al.*, 2019). Regarding enzymes, two FAAH inhibitors also showed inhibitory effects on food-SA (sweetened milk) and reinstatement in rats (Adamczyk *et al.*, 2009). More recently, MAGL-Tg mice in which 2-AG levels are reduced in forebrain exhibited impairments in developing high-fat food-CPP (Wei *et al.*, 2016). Finally, Friemel et al. used an innovative approach entitled "pleasure attenuated startle (PAS)" (Friemel *et al.*, 2014). Briefly, a

conditioned odor is thought to induce a pleasant affective state that attenuates an acoustic startle response (aversive reflex). In this conditioning, WIN55,212 increased while Rimonabant diminished PAS (Friemel *et al.*, 2014). Altogether, the ECS is closely linked to palatable food intake. Therefore, the findings showing that obesity and eating disorders such as AN, BN or BED are associated with *Cnr1* and *Faah* genes polymorphisms in humans (Monteleone *et al.*, 2008, 2009) and *Cnr2* polymorphism in mice (Ishiguro *et al.*, 2010) are not surprising. Overall, studies above confirm that the ECS is a crucial central mediator of palatable food reward.

i. Binge

As a reminder, BED episodes are characterized by a huge amount of food intake within a short period of time. Most studies using a binge-like paradigm investigated the effect of CB1 blockade on palatable food intake. Thus, in a binge-like sucrose paradigm, AM-251 reduced sucrose intake in adolescent mice but not in adults (Agoglia *et al.*, 2016). In rats bingeing on sweet fat, Surinabant (a CB1 antagonist) dose-dependently decreased this behavior (Parylak *et al.*, 2012). Rats with intermittent access to both chow and palatable food exhibited binge-like intake which was dose-dependently reduced by rimonabant (Dore *et al.*, 2014). Mancino et al. used an innovative approach to induce an addictive-like eating behavior in mice. Among the animals, the authors identified a subgroup of animals losing behavioral control representing the pathological continuum from controlled to compulsive use. Interestingly, rimonabant treatment during training period reduced the percentage of mice reaching the addiction-like criteria for this subgroup (Mancino *et al.*, 2015). Altogether, it appears clearly that CB1 blockade decreases not only palatable food intake but also binge-like behaviors. On the other hand, CB1 activation with THC in a margarine binge-eating paradigm increased margarine intake in female rats with access to margarine 2hr/day, while the FAAH inhibitor URB597 showed no effect. Again, rimonabant dose-dependently reduced margarine intake in female rat with access to margarine only three times a week (Scherma *et al.*, 2013).

Overall, even if the role of CB1 is increasingly apparent in the modulation of palatable food intake, the contributing part of CB2 and endocannabinoid enzymes in this process is still blur. Therefore, further studies are needed to clarify their specific role.

2. Food-induced modifications of cannabinoid genes, receptors, endocannabinoid levels

The relationships between the ECS and food intake has been the subject of abundant research. Many studies investigated whether palatable food modulate the ECS in reward-related brain regions.

First, AEA but not 2-AG levels were significantly up-regulated in plasma from women with AN, BN and BED (Monteleone *et al.*, 2005). Very interestingly, both eCBs were up-regulated in plasma of mice which underwent 60 days of western diet (high fat-high sugar diet) (Argueta & DiPatrizio, 2017). Noteworthy, Monteleone et al. recently showed that 2-AG levels are increased 5 min before eating hedonic food suggesting an anticipatory response (Monteleone *et al.*, 2012). Moreover 2-AG levels are also increased after eating the favorite food, whereas they decreased after eating the non-favorite food (Monteleone *et al.*, 2016). In rats with limited access to margarine, AEA levels were decreased in PFC but increased in NAc while 2-AG levels only increased in HPC (Satta *et al.*, 2018). In the same study, CB1 binding was significantly decreased only in cingulate cortex (Satta *et al.*, 2018). One study assessed the effect of three different diets on ECS elements in rats: standard chow diet, high-carbohydrate diet and high-fat diet. The high-fat diet elevated AEA levels in HPC while food restriction from standard diet reduced both eCBs in HPC (Rivera, Luque-Rojas, *et al.*, 2013). Moreover, both carbohydrate and fat diets increased CB1 expression, NAPE-PLD/FAAH and DAGLα/MAGL ratios in HPC (Rivera, Luque-Rojas, *et al.*, 2013). Altogether, these finding point towards a general pattern indicating an increase of eCBs following a palatable food diet even if eCB levels changes in cerebral regions need a more detailed analysis.

Regarding CB1, in food-restricted rats to 2hr food access during two weeks, CB1 protein expression was reduced in PFC only 5min after presentation of food, then return to a normal expression level suggesting a anticipatory role (Dazzi *et al.*, 2014). CB1 gene expression is also reduced in VTA in rats exposed acutely or chronically to a cafeteria diet (Martire *et al.*, 2014). On the other hand, in mice with limited access to a sweet solution for 5 weeks, CB1 gene expression increased in NAc (Soto *et al.*, 2015). Interestingly, palatable food increased dendritic spines in NAc shell (in a similar way to cocaine) and this effect is CB1-dependent (Guegan *et al.*, 2013). In a paradigm of eating addictive-like behavior (according to DSM5 criteria) with chocolate-flavored pellets, mice exhibiting such behavior showed CB1 gene expression increase and decrease, in PFC and NAc, respectively (Mancino *et al.*, 2015). However, in a study investigating the effect of different dietary conditions, only groups with binge and intermittent access to sweetened fat exhibited a decrease of CB1 gene expression in NAc and cingulate cortex (Bello *et al.*, 2012).

Overall, access to a caloric diet, palatable food or sweet solution strongly affects the ECS, especially in PFC and NAc. However, among all these modifications, it is difficult to see a pattern because of the too many different paradigms used. Moreover, studies using cafeteria-like diet could not determine whether the effects observed were induced by sugar or fat alone or both.

In the following paper, we assessed the impact of a binge-like intake of sucrose on the ECS in reward-related brain regions, at the transcriptional and biochemical level. Moreover, we conducted the first transcriptomic analysis following a binge-sucrose paradigm in the NAc of rats.

Transcriptomic analysis of binge sucrose-induced neuroadaptations: a focus on the endocannabinoid system

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Key words: binge eating disorder, sucrose, endocannabinoid system, gene expression, nucleus accumbens

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Abbreviations:

BED: Binge-eating disorder; Cocaine-SA: cocaine self-administration; CPP: compartment place preference; DS: dorsal striatum; ECS: endocannabinoid system; GPCR: G protein coupled receptor; HPC: hippocampus; HYP: hypothalamus; LTP: long-term potentiation; PFC: prefrontal cortex; NAc: Nucleus Accumbens; VTA: ventral tegmental area.

Abstract

Occidental countries currently face an epidemic of obesity and related diseases. As obesity is a complex pathology, therefore we investigated common alterations induced a binge-sucrose paradigm in reward-related brain regions. We focused our research on the endogenous cannabinoid system which modulates palatable food intake. Separate groups were given intermittent (12h) or continuous (24 h) access to a sweet solution (10% sucrose or 0.1% saccharin) and food in their home cage over 28 days. Intermittent sucrose access induced binge-sucrose intake, defined as increased consumption within the first hour. We then analyzed transcriptional regulation and endocannabinoid levels (Anandamide and 2-arachidonoylglycerol) of components of the endocannabinoid system including enzymes and its two receptors, cannabinoid receptors CB1 and CB2 in the prefrontal cortex, nucleus accumbens, dorsal striatum and hippocampus. We found that binge-sucrose intake induced an increase of CB1 gene expression in nucleus accumbens of binge-sucrose animals. We also observed an increase of anandamide in the prefrontal cortex and a decrease of 2-arachidonoyglycerol in the hippocampus of rats given a 12h intermittent access to sucrose. Finally, we conducted an RNA-Seq analysis in the nucleus accumbens of all groups of rats and found gene expression commonalities between excessive sucrose consumption and drugs of abuse. To conclude, our findings shed light on overlapping mechanisms between binge-like intake and drugs addiction. We also highlight several potential new therapeutic target in binge-eating disorder.

Introduction

Since 2013, binge-eating disorder (BED) is recognized as an independent disorder in DSM-5. BED is characterized by uncontrollable episodes of binge eating within a discrete period of time; not followed by compensatory behavior such as purging or physical exercise (American Psychiatric Association, 2013). This is the most frequent eating disorder as most studies estimate the proportion of individuals with BED is 3% (Hudson *et al.*, 2007; Kessler *et al.*, 2013; Solmi *et al.*, 2016). Individuals who binge show significantly higher obesity rates than individuals with no eating disorders (Kessler *et al.*, 2013), thus, binge eating may contribute to the growing obesity epidemic (Stojek & MacKillop, 2017) even though obesity or overweight among individuals with BED is only one third. Since the concept of food addiction and the Yale Food Addiction Scale (Gearhardt *et al.*, 2009), several studies evaluated binge eating as a predictor of addictive traits (Davis *et al.*, 2011; Gearhardt *et al.*, 2012; Burmeister *et al.*, 2013; Clark & Saules, 2013). Thus, further investigations are needed to grasp the etiology and mechanisms that drive BED (Hutson *et al.*, 2018). In that purpose, animal models have been developed to delineate the underlying cognitive and biological mechanisms of BED. For instance, giving alternate access to highly

palatable food and normal chow (Cottone *et al.*, 2008), high-fat food or intermittent access toS sucrose (Avena, Rada, *et al.*, 2008; Corwin *et al.*, 2011) induce binge eating behavior (Iemolo *et al.*, 2012; Rossetti *et al.*, 2014). The binge sucrose paradigm induces release of dopamine in the nucleus accumbens (NAc) shell (Rada *et al.*, 2005). Interestingly, such binge eating behavior can be modulated by the endocannabinoid system (ECS) which is the endogenous system activated by Δ-9-tetrahydrocannabinol from *Cannabis Sativa*. Two receptors, cannabinoid receptor 1 (CB1R) and 2 (CB2R) encoded by *Cnr1* and *Cnr2* respectively, constitute this system along with two majors' endocannabinoids (eCBs) ligands, the anandamide (AEA) and 2-arachidonoyglycerol (2AG). AEA is synthetized by the enzyme NAPE-PLD (*Nape-Pld*) and degraded by FAAH (*Faah*) while 2AG is produced by DAGLα (*Daglα*) and degraded by MAGL (*Mgll*). The ECS is involved in many functions such as memory, learning, reward or pain (Mechoulam & Parker, 2011; Dhopeshwarkar & Mackie, 2014; Mechoulam *et al.*, 2014; Lu & Mackie, 2016). Not only, ECS modulate drug reward, such as cocaine seeking (De Vries *et al.*, 2001; Soria *et al.*, 2005) for instance, but also research group demonstrated the ECS involvement in food intake and obesity (Di Marzo & Matias, 2005; Pagotto *et al.*, 2006; Mazier *et al.*, 2015). It is well known that cannabis promotes feeding (Abel, 1975; Williams *et al.*, 1998; Koch *et al.*, 2015) while CB1R blockade reduces food intake (Arnone *et al.*, 1997; Simiand *et al.*, 1998; Colombo *et al.*, 2002) and binge-eating (Scherma *et al.*, 2013; Dore *et al.*, 2014). Furthermore, release of eCBs in ventral tegmental area induce palatable food intake (Meye & Adan, 2014). More recently, evidence indicate cannabidiol, the second major constituent of *Cannabis Sativa*, reduces sucrose self-administration (Bi *et al.*, 2019).

To further characterize the ECS involvement in food intake and binge-eating, few studies investigate whether binge eating affects CB1R. For instance, CB1R gene expression and availability is decreased in NAc by binge eating (Bello *et al.*, 2012; Blanco-Gandía, Cantacorps, *et al.*, 2017). In prefrontal cortex (PFC), in food-restricted rats to a 2hr period each day during 3 weeks, CB1R expression is decreased simultaneously with feeding-associated increase in dopamine (Dazzi *et al.*, 2014). On the other hand CB1R expression is up-regulated along with a decrease of DNA methylation at CB1R gene promoter region in PFC of addict-like mice (Mancino *et al.*, 2015). These mixed results are probably due to the difference between protocols. A recent study in rats bingeing on palatable food produced very few changes on the ECS, but the authors noticed a decrease of *Faah* expression in hypothalamus (HYP) along with a concomitant decrease of acetylation at lysine 27 of histone 3 at *Faah* promoter (Pucci *et al.*, 2018). Finally, weeks of treatment with either 23% glucose or fructose solution induced no effects on CB1R functionality in reward-related brain regions (Rojo *et al.*, 2014).

Regarding eCBs levels, bingeing on high fat diet decreased AEA levels in dorsal striatum (DS), amygdala and hippocampus (HPC) whereas 2AG levels increased in HPC. Concomitantly, CB1R expression density declined in PFC in rats bingeing on palatable food (Satta *et al.*, 2018). On

the other hand, fasting after palatable food diet increased levels of both AEA and 2AG in limbic forebrain. Moreover, 2AG also increased in HYP but in opposite, decreased in animals eating palatable food (Kirkham *et al.*, 2002). Interestingly, offspring from palatable diet-fed dams showed lower levels of AEA in both HPC and HYP and 2AG in HYP (Ramírez-López *et al.*, 2016). Finally, in patients with BED, AEA level was increased in blood without any effect on 2AG level (Monteleone *et al.*, 2005).

Studies above reveal the eCBs is strongly affected by diet but most of studies above did not investigate the effects of sucrose itself but rather fat or palatable food. Thus, using a validated model of bingeing in which rats have access to sucrose and chow for either 12 or 24 h per day (Avena *et al.*, 2005; Avena, 2007; Avena, Bocarsly, *et al.*, 2008; Avena, Rada, *et al.*, 2008), the first goal of our study was to examine whether sucrose bingeing leads to ECS modifications on transcriptional and biochemical levels. Regarding the addictive potential of sucrose, we hypothesized that such paradigm could produce strong changes in NAc, therefore, our second objective was to decipher the global transcriptomic adaptations in NAc induce by sucrose binge intake.

Materials and Methods

Subjects

91 male Wistar rats (Charles River, Canada and Janvier Laboratories, France) weighting 250–300 g at the beginning of the tests were habituated for two weeks to housing conditions in a temperature and humidity-controlled environment with a reverse 12 h light/dark cycle (lights OFF at 4:00 AM) in standard polycarbonate home cage. Rats were group housed in standard cages with *ad libitum* access to food and water until 1 day before sucrose experiments. All animals were then single housed to measure specific intake of each subject. All experimental procedures were performed according to the European Union laws for animal studies and approved by the institutional ethics committee CREMEAS (Comité d'Éthique pour l'Expérimentation Animale de Strasboug, France). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Sucrose bingeing procedure

The protocol for compulsive sucrose consumption was based on a protocol inducing sucrose bingeing (Avena, Rada, *et al.*, 2008; Maracle *et al.*, 2019). Animals were randomly assigned to one of four groups: intermittent sucrose (12h access to sucrose and food) (n=28), intermittent saccharin (12h access to saccharin and food) (n=8), intermittent food (12h access to food) (n=28), or ad libitum sucrose (24h access to sucrose and food) (n=27). For 28 days, 4h after active cycle start, animals were weighed and presented with solution and food. For the intermittent access groups, solution intake (ml) was measured 1h after presentation and on removal (12h post

presentation). For animals in the ad libitum group (24h sucrose), solution intake was measured 1, 12, and 24h after presentation. Food intake (g) was measured at the end of the access period (12 or 24 h). Sucrose and food consumption were measured as solution (ml) and grams (g) consumed per body weight (g) after the first hour of access for sucrose, and at the end of 12 or 24 h for both. Next day after last session, animals were given an overdose of pentobarbital (40 mg/kg, i.v) followed by decapitation to perform brain extraction. We were careful to execute all extraction procedures in less than 30min to avoid increased levels of AEA (Schmid et al., 1995).

Quantitative real-time PCR

Brain structures of interest were collected by punches. Samples were immediately frozen on dry ice and kept at -80°C. Total RNA was extracted using Ribozol (VWR) according to manufacturer's instructions. RNA quality (260/280 ratio: 1.8–2) and quantity was measured with a NanoVue™ (GE healthcare) spectrophotometer. Reverse transcription to obtained cDNA was performed on 750 ng of total RNA in a 20 µL final volume, with iScript (iScript™ cDNA Synthesis Kit, Biorad, France). Real-time PCR was performed using a CFX96 Touch™ apparatus (Biorad, France) and Sso Advanced™ Universal SYBR Green supermix (Biorad, France) in a final volume of 15 µl. Thermal cycling parameters were 30 sec at 95°C followed by 40 amplification cycles of 5 sec at 95°C and 45 sec at 60°C. Primer sequences for all tested genes are given in **Table 1**. Expression levels were normalized to *Rplp0* housekeeping gene levels and compared between controls and treated samples using the 2-ΔΔCt method {Livak, 2001 #61}.

Mass spectrometry

Preparation of Tissues

PFC, HIPP, NAc and DS tissues were sonicated with a Vibra Cell apparatus (2 times 5 s, 90W; Sonics, Newtown, U.S.A.) in 200 µl of H_2O. The homogenate was centrifuged (20,000g, 30 min, 4°C) and the supernatant was recovered. Protein concentration was determined using the Bradford method (Protein Assay, Bio-Rad, Marnes-la-Coquette, France). 150 µl of the supernatant were taken and mixed with 50 µl of acetonitrile (ACN) 100% containing known fixed amounts of deuterated internal standards containing 400.26 pmol of D8-2AG (ref sc-480539; Santa Cruz, Heidelberg, Germany) and 100.15 pmol of D4-AEA (Tocris/Biotechne, Lille, France). The addition of heavy compounds allows to perform a quantification using isotopic dilution. The sample was centrifuged (20,000g for 30 min, 4°C) and the supernatant was collected and evaporated to dryness. Samples were re-suspended in 20 µl of ACN 30% / H_2O 69.9% / formic acid 0.1% (v/v/v)

LC-MS/MS Instrumentation and Analytical Conditions

Analyses were performed on a Dionex Ultimate 3000 HPLC system (Thermo Scientific, San Jose, USA) coupled with a triple quadrupole Endura mass spectrometer (Thermo Scientific). The

system was controlled by Xcalibur v. 2.0 software (Thermo Electron). Samples (3µl/µl) were loaded onto a microbore C18 ODS column (1x100 mm, 3 µm UniJet microbore ODS, ref MF8949, BioAnalytical Systems Inc., West Lafayette, U.S.A.) heated at 40°C. The presence of 2-AG, AEA, D8-2-AG and D5-AEA was studied using the multiple reaction monitoring mode (MRM). Elution was performed at a flow rate of 50 µl/min by applying a linear gradient of mobile phases A/B. Mobile phase A corresponded to ACN 1% / H_2O 98.9% / formic acid 0.1% (v/v/v), whereas mobile phase B was ACN 99.9% / formic acid 0.1% (v/v). The gradient used is detailed in **supplemental table 1**.

Electrospray ionization was achieved in the positive mode with the spray voltage set at 3,500 V. Nitrogen was used as the nebulizer gas. Desolvation (nitrogen) sheath gas was set to 10 Arb and Aux gas was set to 5 Arb. The Ion transfer tube was heated at 287°C. Q1 and Q2 resolutions were set at 0.7 FWHM, whereas collision gas (CID, argon) was set to 2 mTorr. Identification of the compounds was based on precursor ion, selective fragment ions and retention times obtained for 2-AG, AEA, D8-2-AG and D5-AEA. Selection of the monitored transitions and optimization of collision energy and RF Lens parameters were manually determined (see **supplemental table 2**). Qualification and quantification were performed in MRM mode. Quantification was obtained using Quan Browser software (Thermo Scientific). For tissues and fluids, alkaloids were quantified using calibration curves of external standards added to brain extract of naive mice and submitted to the same procedure described for respective fluids and tissue recovery. All amounts of endocannabinoids measured in samples fit within the standard curve limits, with typical analytical ranges (the range of amounts that can be accurately quantified) from 1 fmol – 100 pmol to 150 fmol – 100 pmol. Precision (CV% between repeated injections of the same sample) values were <1% for same-day measurements and <5% for inter-day measurements. The amount of 2AG (nmol) and AEA (pmol) observed were normalized according to protein concentrations (mg).

RNA-Seq analysis

Total RNA from NAc tissues was extracted and purified with Nucleospin® RNA kit (Macherey-Nagel, France). RNA-Seq libraries were generated from 500 ng of total RNA using TruSeq Stranded mRNA LT Sample Preparation Kit (Illumina, San Diego, CA), according to manufacturer's instructions. Briefly, following purification with poly-T oligo attached magnetic beads, the mRNA was fragmented using divalent cations at 94oC for 2 minutes. The cleaved RNA fragments were copied into first strand cDNA using reverse transcriptase and random primers. Strand specificity was achieved by replacing dTTP with dUTP during second strand cDNA synthesis using DNA Polymerase I and RNase H. Following addition of a single 'A' base and subsequent ligation of the adapter on double stranded cDNA fragments, the products were purified and enriched with PCR (30 sec at 98oC; [10 sec at 98oC, 30 sec at 60oC, 30 sec at 72oC] x 12 cycles; 5 min at 72oC) to create the cDNA library. Surplus PCR primers were further removed by

purification using AMPure XP beads (Beckman-Coulter, Villepinte, France) and the final cDNA libraries were checked for quality and quantified using capillary electrophoresis. Reads were preprocessed in order to remove adapter, polyA and low-quality sequences (Phred quality score below 20). After this preprocessing, reads shorter than 40 bases were discarded for further analysis. These preprocessing steps were performed using cutadapt (Martin, 2011) version 1.10. Reads were mapped to rRNA and spike sequences using bowtie (Langmead & Salzberg, 2012) version 2.2.8 and reads mapping to rRNA or spike sequences were removed for further analysis. Reads were mapped onto the rn6 assembly of Rattus norvegicus genome using STAR (Dobin *et al.*, 2013) version 2.5.3a. Gene expression quantification was performed from uniquely aligned reads using htseq-count (Anders *et al.*, 2015) version 0.6.1p1. Only non-ambiguously assigned reads have been retained for further analyses. Comparisons of interest were performed using the test for differential expression proposed by Love et al. (Love *et al.*, 2014) and implemented in the Bioconductor package DESeq2 version 1.16.1. P-values were adjusted for multiple testing using the Benjamini and Hochberg method (Benjamini & Hochberg, 1995).

In order to study the biological functions and pathways regulated by sucrose intake, transcripts significantly regulated ($p < 0.05$) were analyzed with WEB-based Gene Set Analysis Toolkit (WebGestalt (http://www.webgestalt.org/)). We then used candidate gene prioritization with Toppgene (https://toppgene.cchmc.org/) in order to study the involvement of the differentially expressed genes in addiction, obesity and neuroinflammation. Training list were downloaded from OMIM(https://www.omim.org/).

Statistical analysis

All results are expressed as mean ± sem. Behavioral experiments were analyzed using repeated-measures analysis of variance (RM ANOVA) with time as repeated measures and group as the between-subjects measure. All other experiments were analyzed using one-way ANOVA (treatment). Bonferroni *post hoc* test was applied to analyze significant interaction. Significance was set at $p \leq 0.05$.

Results

Sucrose consumption

The 12hr sucrose group consumed approximately 6% of their body weight in the first hour. This would correspond to 18ml of sucrose consumed in 1h for a 300g rat. During the first hour of access, the difference between group was significant **(Figure 1A)** [$F(1.028;3.085) = 458.7$ $p=0.0002$]. This reveals that the 12hr sucrose group consumed more solution than the 12hr saccharin or 24hr sucrose groups. On a daily scale, the two sucrose groups showed similar patterns of escalation and overall intake as previously described (Maracle et al., 2019) **(Figure**

1B). The difference between group was again significant [F (1.314;3.91) =665.2 p<0.0001]. As previously demonstrated (Maracle *et al.*, 2019), daily food intake was lower in both sucrose group compare to the 12hr food and 12hr saccharin group along with a significant difference between groups **(Figure 1C)** [F (1.043;3.129) =24.91 p<0.0138]. The weight of the 24hr sucrose group was heavier compare to all other groups during the experiment **(Figure 1D)** [F (1.041;3.124) =30.90 p<0.0102].

Gene expression of ECS elements

We analyzed compulsive sucrose intake effect on *Cnr1*, *Cnr2*, *Nape-Pld*, *Faah*, *Daglα* and *Mgll* expression in PFC, NAc, DS and HPC. We observed an increase of *Cnr1* in NAc in the 12hr sucrose group compared to both control and 24hr sucrose groups **(Figure 2A)**. (*NAc*: F (2,24) = 4.901, *p* = 0.0164). There were no effects in other structures **(Figure 2A)** [***Cnr1***: (*PFC*: (F (2, 20) = 0.8424, *p* = 0.4454), (*DS*: F (2, 21) = 0.04124, *p* = 0.9597), (*HPC*: F (2, 20) = 1.363, *p* = 0.2786)]. There was no effects on *Cnr2* expression either **(Figure 2B)** [***Cnr2***: (*PFC*: (F (2, 20) = 0.7745, *p* = 0.4743), (*NAc*: F (2, 15) = 0.4434, *p* = 0.65), (*DS*: F (2, 19) = 1.269, *p* = 0.3039), (*HPC*: F (2, 25) = 1.257, *p* = 0.3019)].

Regarding enzymes gene expression, *Faah* expression was decreased in NAc only in the 24hr sucrose group **(Figure 2C)** [***Faah***: (*NAc*: F (2, 21) = 4.033, *p* = 0.0330)] while *Daglα* expression decreased in both sucrose groups in NAc **(Figure 2D)** [***Daglα***: (*NAc*: F (2, 21) = 10.22, *p* = 0.0008)]. We noticed no other effect on enzymes in any of the structures of interest **(Figure 2C,D)**.

Endocannabinoid levels

Regarding gene expression modulation of the enzymes, we hypothesized that sucrose compulsive intake would regulate endocannabinoids levels. Using LC-MS/MS, we measured endocannabinoids levels in PFC, NAc, DS and HPC. We observed an increase of AEA in PFC without any other changes in remaining structures **(Figure 3A)** [***AEA***: (*PFC*: (F (2, 21) = 4.053, *p* = 0.0325), (*NAc*: F (2, 21) = 0.04061, *p* = 0.9603), (*DS*: F (2, 21) = 0.1651, *p* = 0.8489), (*HPC*: F (2, 18) = 0.1347, *p* = 0.8749)]. On the opposite, we measured a decrease of 2AG in HPC **(Figure 3B)** [***2AG***: (*PFC*: (F (2, 21) = 0.4915, *p* = 0.6186), (*NAc*: F (2, 21) = 0.204, *p* = 0.8170), (*DS*: F (2, 21) = 0.2154, *p* = 0.8080), (*HPC*: F (2, 18) = 3.752, *p* = 0.0434)]. Interestingly, both regulations only occurred in the 12hr sucrose group.

Compulsive sucrose intake effect on NAc transcriptome

To characterize the transcriptome of rat NAc, the differential analysis for changes in gene expression was run with three comparisons: “Control vs 12hr sucrose”, “Controls vs 24hr sucrose” and “12hr sucrose vs 24hr sucrose”. In the condition “Control vs 12hr sucrose”, we identified only 1 differentially regulated gene, *Mc4r* encoding the melanocortin receptor 4, which was down

regulated. Regarding the comparison "Control vs 24hr sucrose", we identified 342 differentially expressed genes (214 increased, 128 decreased) **(supplemental table 3)**. Noteworthy, *Mc4r* expression was also decreased in this comparison. Within the last comparison "12hr sucrose vs 24hr sucrose", we observed only 1 differentially regulated gene, *Mblac2* encoding the metallo-β-lactamase domain containing 2. The 342 differentially expressed genes from "Control vs 24hr sucrose" were subjected to gene ontology (GO) classification using the WebGestalt gene analysis tool using the overrepresentation enrichment analysis tool. Top 10 of GO terms for biological process, cellular component and molecular function are represented in **Table 2**. Genes were enriched in processes that regulate cell adhesion and growth; cell and neuron projection components; in function regulating lipid, phospholipid and carbohydrate binding.

In a subsequent analysis, we then subjected the 342 genes to a network topology-based analysis with biological general repository for interaction datasets (PPI BioGRID) and top 400 ranking neighbors using WebGestalt. A network was then generated and clustered using Cytoscape 3.7.1 (https://cytoscape.org/) and ClusterMaker - MCL Cluster plugin tool **(Supplemental figure 1)**. We obtained 11 clusters, each subjected to a BINGO (Biological Networks Gene Ontology) plugin tool which confirmed previous findings and revealed an involvement in "response to cocaine" [GO-term: 42220 ; adjusted p-value: 5.6593E-10] with the genes GRIA1, GRIN2A, HOMER1, CDK5, DPYSL2, MDM2, NCAM1, DRD2, GRIN2B, SLC6A3, SNCA and "response to drug" [GO-term: 42493 ; adjusted p-value: 0.0018853] with the following genes GRIA1, PRKCB, GAD2, PARK7, HMGB1, YWHAZ, SLC6A3, PARK2, SLC8A1, LDHA, GRIN2A, FABP4, DPYSL2, MYO6, MDM2.

Candidate gene prioritization analysis

To better characterize gene expression changes in the "Control vs 24hr sucrose" condition, we subjected the differentially expressed genes from our analysis against current literature. Considering previous results and literature, we confronted our gene list versus training lists with genes involved in "addiction"(Lenoir *et al.*, 2007; Avena, Rada, *et al.*, 2008; Westwater *et al.*, 2016; DiNicolantonio *et al.*, 2018), "obesity" (Small, 2009; Volkow *et al.*, 2011) and "neuroinflammation" (Hsu *et al.*, 2015; Gao *et al.*, 2017; Cigliano *et al.*, 2018) obtained from OMIM (https://www.omim.org/). Using the candidate gene prioritization tool from Toppgene (https://toppgene.cchmc.org/), we looked at the overall p-value considering the following factors: molecular function, biological process, cellular component, pathway, pubmed and disease. We identified 11, 10 and 5 candidate genes for "addiction", "obesity" and "neuroinflammation" respectively **(Figure 4)**. 4 genes were in common between the three analysis, GRIA1 (Glutamate receptor 1), FYN (Tyrsosine-protein kinase Fyn), NOS3 (Nitric oxide synthase, endothelial), RHOA (Transforming protein RhoA) **(Figure 4)**.

Discussion

Sucrose consumption

As previously demonstrated (Avena *et al.*, 2005; Smail-Crevier *et al.*, 2018; Maracle *et al.*, 2019) intermittent access to sucrose induce bingeing behavior in animals during the first hour access. This behavior was stable and maintained across 28 days. As in Maracle et al. (Maracle *et al.*, 2019), animals getting saccharin access did not display bingeing behavior in opposite to a study conducted in mice with lower concentration of saccharin (Yasoshima & Shimura, 2015).

ECS gene expression

Sucrose consumption displayed only few modifications on ECS elements gene expression. CB1R gene expression was increased in NAc only in the 12hr-sucrose group with limited access to sucrose. This finding is noteworthy as CB1R in NAc modulate reward. Indeed, eCBs induce dopamine release in NAc shell (Solinas *et al.*, 2006) as well as CB1R activation in NAc (Sperlágh *et al.*, 2009). Several evidences indicate that both CB1R and eCBs in NAc also modulate behavior such as social reward (Trezza & Vanderschuren, 2008; Trezza *et al.*, 2012; Wei *et al.*, 2015; Manduca *et al.*, 2016), food (Hernandez & Cheer, 2012) or morphine reward (Khaleghzadeh-Ahangar & Haghparast, 2015) accompanied by modulation of neuronal activity in NAc (Grueter *et al.*, 2010; Hernandez & Cheer, 2012). Two previous studies relying on bingeing of palatable food found a decrease of CB1R gene expression in NAc (Bello *et al.*, 2012; Blanco-Gandía, Cantacorps, *et al.*, 2017). Therefore, the increase we observed in our conditions may indicate a specific sucrose-induced adaptation. Interestingly, exposition to both peripubertal stress and play fighting in rats also increase CB1R gene expression in NAc. Following a high fat diet, mice exposed to withdrawal exhibited both CB1R expression decrease in NAc and increase of anxiety (Blanco-Gandía, Aracil-Fernández, *et al.*, 2017). As sucrose bingeing increase anxiety (Avena, Bocarsly, *et al.*, 2008), further studies should confirmed whether CB1R gene expression level is specific to stress or sucrose bingeing. Furthermore, determine whether the ECS could shift sucrose bingeing will bring important clues about its role and mechanisms. Indeed, a CB1R antagonist, SR147778, reduced binge intake of fat (Parylak *et al.*, 2012) as did SR141716 (Rimonabant) with palatable food (Foltin & Haney, 2007). Thus, one could expect a same effect with binge intake of sucrose. To go further, CB1R gene expression is decreased in blood from patients with eating disorders (Frieling *et al.*, 2009). Interestingly, there is a negative correlation between CB1R availability and body mass index in reward-related brain regions. as assessed by PET (Ceccarini *et al.*, 2016). Therefore, further work is needed to evaluate whether CB1R expression or functionality is specifically modulated by sucrose in brain.

Enzymes expression & eCBs levels

We observed only few sucrose-induced changes on ECS enzymes expression. As changes were observed in NAc, we were expecting eCBs levels regulations in this region. Despite no eCBs modulation in NAc, we noteworthy observed eCBs regulations in both PFC and HPC only in the 12hr sucrose group with limited access to sucrose. Therefore, eCBs levels in both regions are most likely modulated by the binge intake of sucrose. Regarding AEA increase in PFC, such modulation in PFC could lead towards an anxiolytic behavior as animals spend more times in open arms of an elevated plus maze in a CB1 dependent-manner (Bortolato *et al.*, 2006; Rubino *et al.*, 2008). Moreover, as withdrawal from sucrose-bingeing increase anxiety (Avena, Bocarsly, *et al.*, 2008), the AEA increase within PFC could act as a stress modulator. Indeed, inhibition of AEA hydrolysis modulate serotoninergic, noradrenergic and GABAergic pathways within PFC (McLaughlin *et al.*, 2012; Bedse *et al.*, 2015; de Morais *et al.*, 2016). These findings suggest AEA modulation of these different pathways regulate stress behavior. In regards to 2AG decrease in HPC, such reduction could blunt social or natural reward (Wei *et al.*, 2016). Furthermore, a 2AG hydrolysis inhibitor alters memory performance (Griebel *et al.*, 2015), further investigations are needed not only to assess whether rats bingeing on sucrose present memory tasks disturbances but also to determine whether sucrose reward is altered after 4 weeks of sucrose bingeing.

Binge sucrose-induced transcriptomic adaptations in NAc

We noticed a decrease of *Mc4r* expression in both sucrose groups compare to controls. MC4R is well known for its role in regulating cardiovascular function, glucose homeostasis and energy balance (Balthasar *et al.*, 2005; Rossi *et al.*, 2011; do Carmo *et al.*, 2013; Sohn *et al.*, 2013; Zechner *et al.*, 2013; Berglund *et al.*, 2014). Indeed, MC4R deficiency is associated with obesity (Huszar *et al.*, 1997; Farooqi *et al.*, 2003; Balthasar *et al.*, 2005; Berglund *et al.*, 2014), hyperglycemia and hyperinsulinemia (Zechner *et al.*, 2013; Berglund *et al.*, 2014). Moreover, MC4R activation is linked to the modulation of reward. Indeed, MC4R blockade blocks the reinforcing and locomotor effects of cocaine (Hsu *et al.*, 2005) or alcohol intake (Shelkar *et al.*, 2015).

Interestingly, our binge sucrose protocol reduces the rewarding value of sucrose in rats (Smail-Crevier *et al.*, 2018). Furthermore, previous work points out NAc-MC4R on D1-medium spiny neurons as regulators of stress-induced anhedonia (Lim *et al.*, 2012). Overall, we propose that *Mc4r* down-regulation observed in our conditions should increase the strength of excitatory synapses on D1 receptor-expressing nucleus accumbens medium spiny neurons due to the decrease of MC4R. Further work should confirm MC4R expression or functionality may be altered by binge sucrose intake in NAc. MC4R gene expression alterations may be due to epigenetic modifications. In offspring of HFD-fed dams, *Mc4r* expression reduction is combined with increase of H3K27Ac at *Mc4r* promoter without any effect on DNA methylation in HYP (Tabachnik *et al.*, 2017). In children, methylation status of *Mc4r* negatively correlated with the BMI from children

with high levels of triglyceride (Kwon *et al.*, 2019). Therefore, further studies should assess whether DNA methylation or histone modifications are linked with alterations of *Mc4r* expression due to binge sucrose. Using recent epigenome editing-based systems, such as zinc finger proteins, transcription activator-like effectors or short palindromic repeats Cas9, to explore precise epigenetic modifications holds great promise (Thakore *et al.*, 2016).

In a subsequent analysis we identified GRIA1, FYN, NOS3 and RHOA as genes involved all at once in addiction, obesity and neuroinflammation. These findings highlight new similarities between drugs of abuse-induced adaptations and food addiction, in our case, sucrose bingeing. Notably, there is extensive literature about GRIA1 and its role in addiction (Zhao *et al.*, 2014; Eisenhardt *et al.*, 2015; Hou *et al.*, 2015; Meyers *et al.*, 2015; Weber *et al.*, 2015; Egervari *et al.*, 2017; Caffino *et al.*, 2018) but few regarding food or sucrose intake (Grillo *et al.*, 2011; Tukey *et al.*, 2013; Peng *et al.*, 2015; Ross *et al.*, 2019). Therefore, we propose further studies investigating binge-like intake of fat or sweet should focus on GRIA1. For instance, overexpression of *Gria1* or inhibition of one of its interacting protein, SAP97, in NAc subregions attenuates cocaine seeking (White *et al.*, 2016). Therefore, as *Gria1* has a potential involvement in palatability and anhedonia (Austen *et al.*, 2017) one could expect similar results regarding sucrose bingeing.

To conclude, our findings bring new clues towards the understanding of overlapping mechanisms between food and drugs addiction (Westwater *et al.*, 2016) and highlight potential new biomarkers in BED.

References

The references are available at the end of the thesis manuscript.

Table 1

Target	Primer sens	Primer antisens
Rplp0	CTGCCCGAGCCGGTGCCATC	TTCAATGGTACCTCTGGAG
Cnr1	TCTGCTTGCGATCATCATGGTGT	AGATGATGGGGTTCACGGTC
Cnr2	AATGGCGGCTTGGATTCAA	TAGAGCACAGCCACGTTCTC
Nape-Pld	AGAGATCCGTGGCGATTCAC	ATCGTGACTCTCCGTGCTTC
Daglα	GGCATGGTACTCTCAGCTGA	GAGGAAGGAGAGAATGGCGG
Faah	CCCCAGAGGCTGTGTTCTTT	GTCAGATAGGAGGTCACGCA
Mgll	GTTGAAGAGGCTGGACATGC	TCACGTGCTGCAACAAATCT

Figure 1

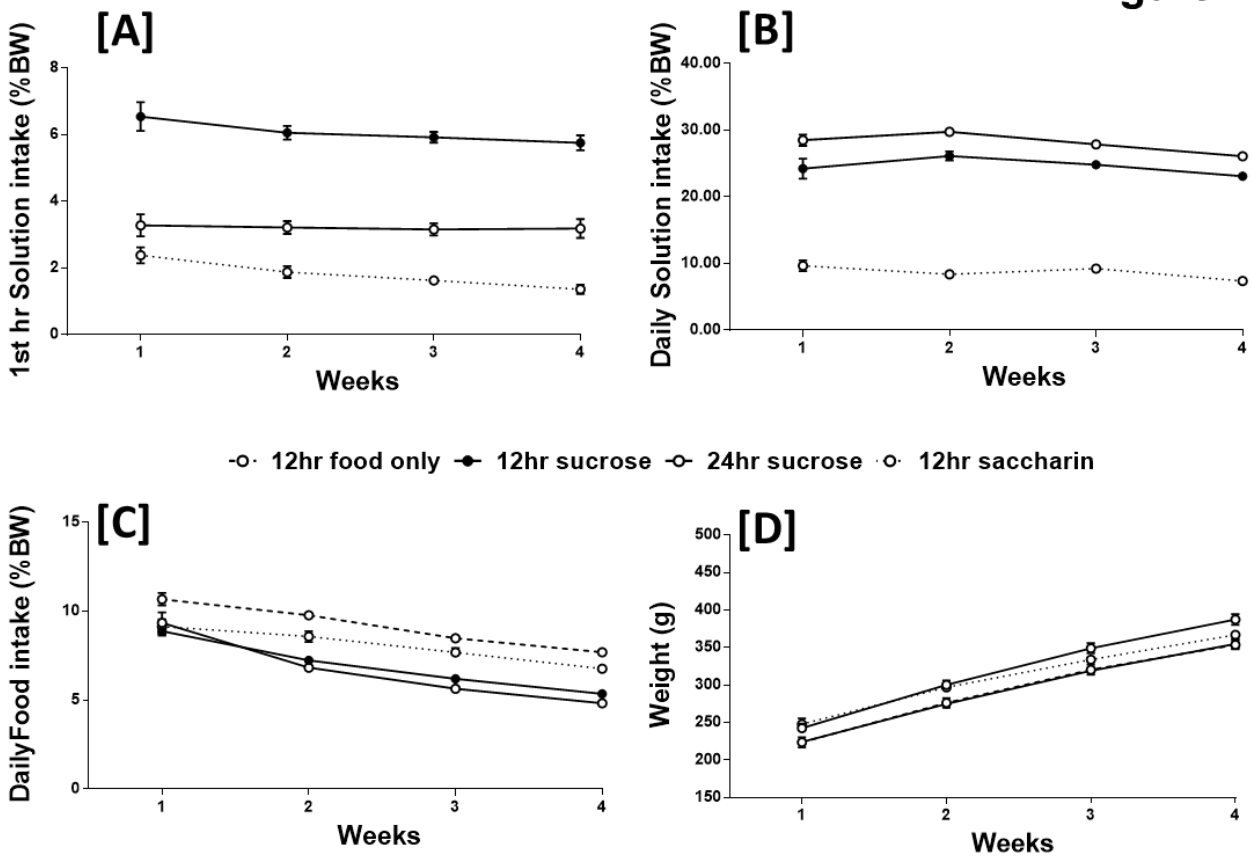


FIGURE 1: Solution and food intake across 4 weeks for each group. Data are presented as group means and error bars represent standard error of the mean **[A]** Mean solution intake during first hour of intake **[B]** Mean solution of the daily intake **[C]** Mean food daily intake **[D]** Mean body weight

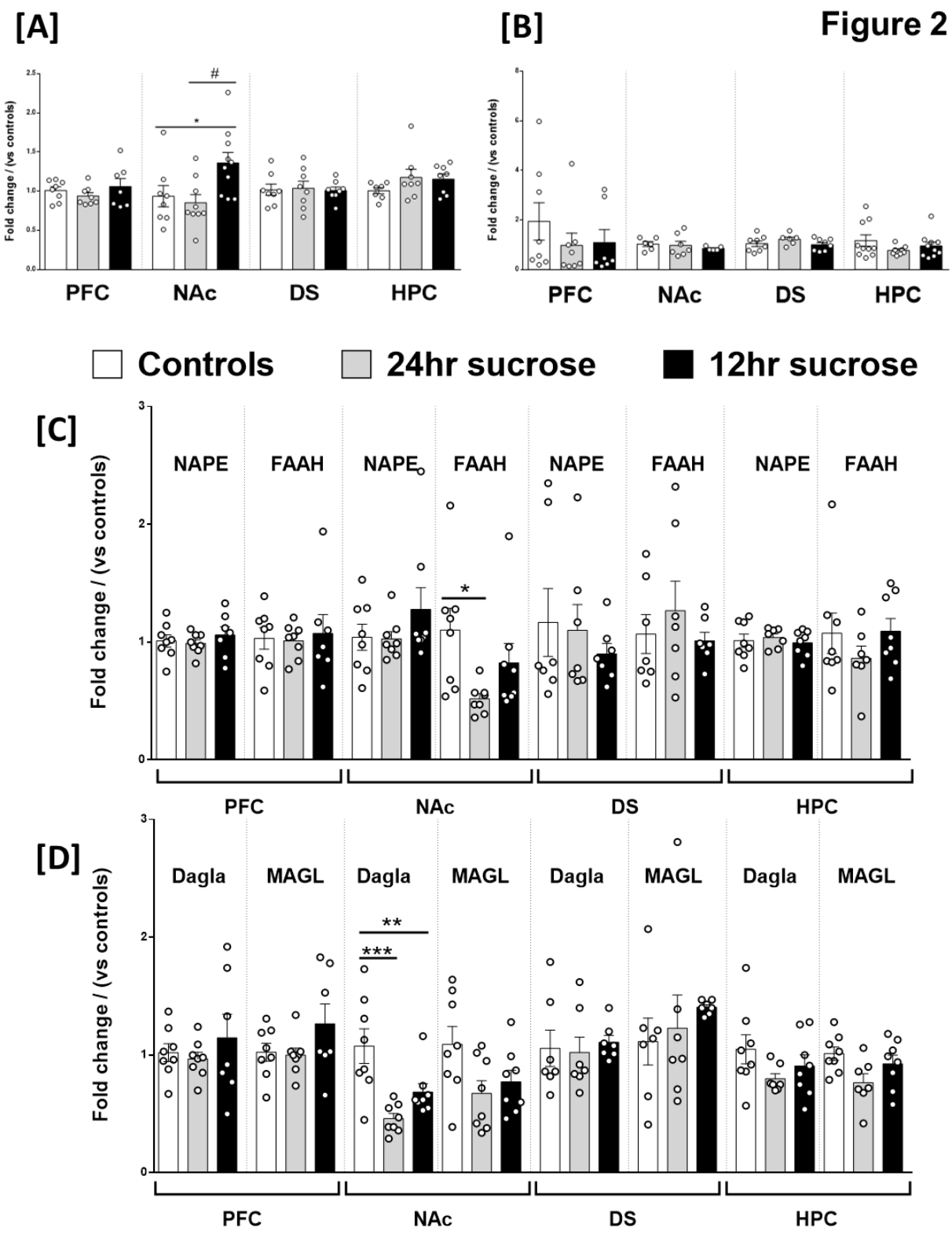


FIGURE 2: ECS elements gene expression after sucrose compulsive intake. Data are presented as group means and error bars represent standard error of the mean **[A]** CB1 gene expression **[B]** CB2 gene expression **[C]** AEA enzymes gene expression **[D]** 2AG enzymes gene expression

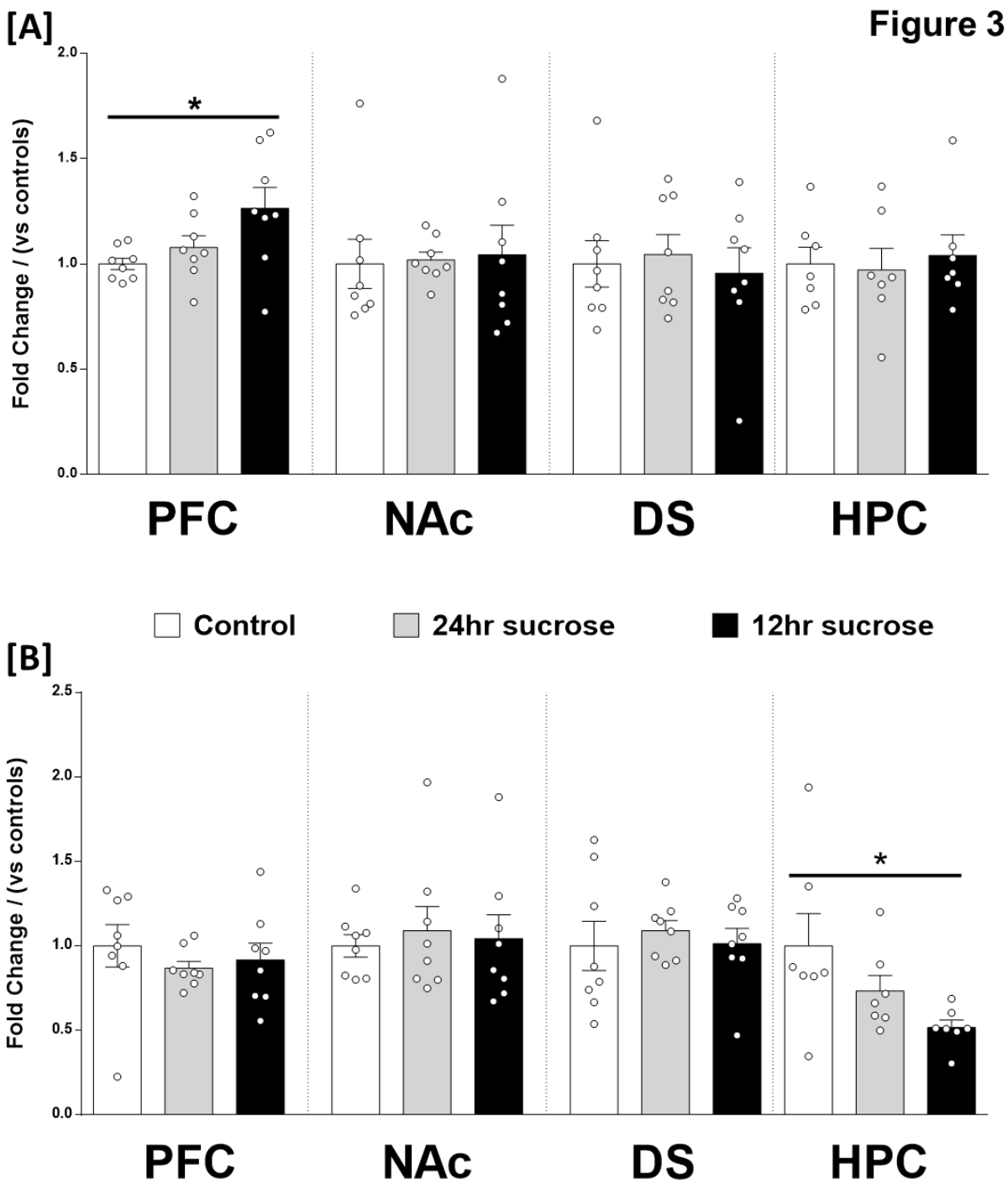


FIGURE 3: AEA and 2AG levels in PFC, NAc, DS and HPC. Data are presented as group means and error bars represent standard error of the mean **[A]** AEA level is increased in PFC in the 12hr sucrose group **[B]** 2AG level is decreased in HPC in the 12hr sucrose group

Table 2

Biological process

Gene Set	Description	Ratio	P Value	FDR
GO:0007160	cell-matrix adhesion	5.2553	0.0000029910	0.020404
GO:0007155	cell adhesion	2.2916	0.0000068835	0.020404
GO:0022610	biological adhesion	2.2771	0.0000078659	0.020404
GO:0034440	lipid oxidation	5.8885	0.000021112	0.041073
GO:0031589	cell-substrate adhesion	3.6376	0.000032845	0.051121
GO:0009612	response to mechanical stimulus	3.5198	0.000047170	0.054016
GO:0001558	regulation of cell growth	3.0368	0.000048588	0.054016
GO:0044242	cellular lipid catabolic process	4.1773	0.000067178	0.059396
GO:0009062	fatty acid catabolic process	5.7925	0.000068693	0.059396
GO:0016054	organic acid catabolic process	3.6610	0.00011316	0.077247

Cellular component

Gene Set	Description	Ratio	P Value	FDR
GO:0042995	cell projection	2.1194	3.1673e-8	0.000032972
GO:0031012	extracellular matrix	4.9145	6.9727e-8	0.000036293
GO:0120025	plasma membrane bounded cell projection	2.0523	2.4046e-7	0.000083441
GO:0044459	plasma membrane part	1.9150	9.2806e-7	0.00021434
GO:0043005	neuron projection	2.2334	0.0000010295	0.00021434
GO:0062023	collagen-containing extracellular matrix	6.0211	0.0000021437	0.00032550
GO:0097458	neuron part	2.0223	0.0000021888	0.00032550
GO:0001725	stress fiber	10.871	0.0000031144	0.00036023
GO:0097517	contractile actin filament bundle	10.871	0.0000031144	0.00036023
GO:0098805	whole membrane	2.2911	0.0000044125	0.00045934

Molecular function

Gene Set	Description	Ratio	P Value	FDR
GO:0008289	lipid binding	2.3895	0.000098660	0.14134
GO:0005543	phospholipid binding	2.8373	0.00017635	0.14134
GO:0008191	metalloendopeptidase inhibitor activity	18.147	0.00047361	0.25307
GO:0097367	carbohydrate derivative binding	1.5785	0.00089692	0.35944
GO:0005539	glycosaminoglycan binding	3.4025	0.0024837	0.54501
GO:0016406	carnitine O-acyltransferase activity	21.776	0.0032392	0.54501
GO:0004064	arylesterase activity	21.776	0.0032392	0.54501
GO:0035620	ceramide transporter activity	21.776	0.0032392	0.54501
GO:0036094	small molecule binding	1.4498	0.0033795	0.54501
GO:0016709	oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen, NAD(P)H as one donor, and incorporation of one atom of oxygen	6.2217	0.0037160	0.54501

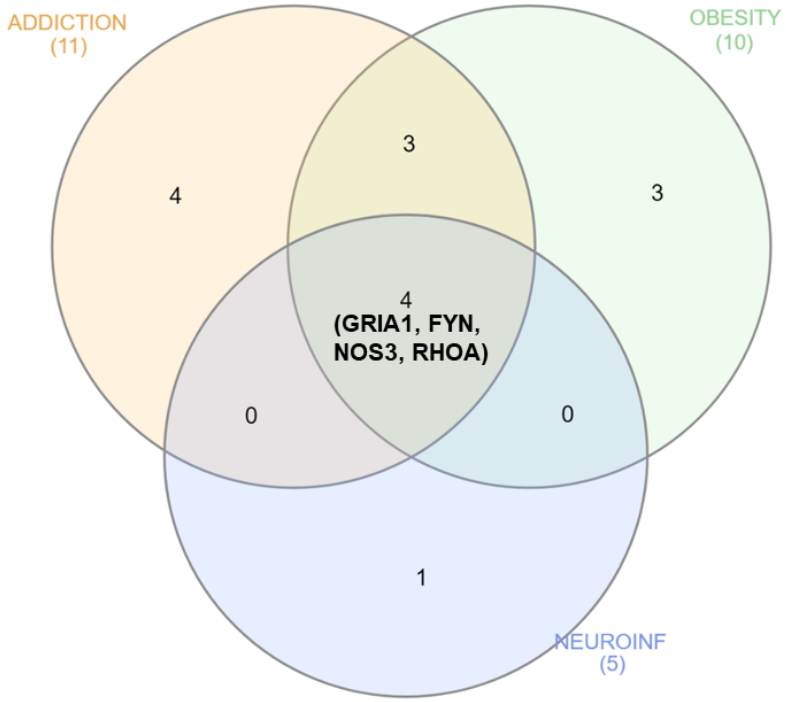
Figure 4

ADDICTION

Rank	GeneSymbol
1	GRIA1
2	ITGB1
3	FYN
4	HCN2
5	TGFB1
6	NOS3
7	ATP1A2
8	GAD2
9	RHOA
10	MC4R
11	TEK

OBESITY

Rank	GeneSymbol
1	ITGB1
2	GRIA1
3	FYN
4	NOS3
5	ATP1A2
6	CLU
7	L1CAM
8	TEK
9	RHOA
10	VEGFB



NEUROINFLAMMATION

Rank	GeneSymbol
1	RHOA
2	GRIA1
3	DGKG
4	FYN
5	NOS3

Supplemental table 1

HPLC gradient

Time (min)	0	2.5	12.5	14.5	15	19
% B mobile phase	55	55	99	99	55	55

Supplemental table 2

MS ionization, selection, fragmentation and identification parameters

Compound	Polarity	Precursor (m/z)	Product (m/z)	Ion product type	Collision Energy (V)	RF Lens (V)
2-AG	Positive	379.28	269.15	Qualification	16.6	164
			287.15	Qualification	14.6	
			361.22	Quantification	14.6	
D8-2-AG	Positive	373.28	209.17	Qualification	26.5	178
			294.33	Qualification	43.2	
			331.04	Quantification	39.0	
AEA	Positive	348.27	203.17	Qualification	17.0	207
			269.17	Qualification	17.1	
			287.15	Quantification	14.8	
D5-AEA	Positive	352.27	203.17	Qualification	17.4	208
			269.17	Qualification	17.1	
			287.15	Quantification	14.9	

IV. Article 4: activation of cannabinoid CB2 receptors induces expression of the epigenetic factors MeCP2 and HDAC2 in rat striatum

This work involved a previous postdoctoral researcher in our lab. I participated in the study by completing some molecular experiments. This works aimed at examining the impact of CB2 activation on the expression of epigenetic factors

Activation of Cannabinoid CB2 receptors induces expression of the epigenetic factors MeCP2 and HDAC2 in rat striatum

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Running title: CB2R stimulation induces MeCP2 and HDAC2 epigenetic factors

Keywords: cannabinoid receptor, neuroepigenetics, striatum, JWH133

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Abbreviations: Ac-H3: histone H3 acetylated at K9/K13; **Ac-H4**: histone H4 acetylated at K5/K8/K12/K16; **eCB**: endocannabinoid; **FAAH**: fatty-acid amide hydrolase; **HDAC**: histone deacetylase; **MeCP2**: methyl-binding protein 2; **TRPV1**: transient receptor potential cation channel subfamily V member 1/ vanilloid receptor 1.

ABSTRACT

The long-term neuroplasticity underlying drug addiction involves emerging epigenetic processes. The endocannabinoid system plays a major role in neuronal plasticity but there is no evidence so far showing that it participates to the epigenetic regulation controlling gene transcription in the brain. We report here that treatment with the anandamide transporter inhibitor, AM404, was able to modify the expression of methyl-CpG-binding protein MeCP2 and histone deacetylase 2 (HDAC2) in several dopaminergic projections regions of rat brain. Protein levels were found to be induced in the striatum in response to a single, but not a repeated treatment (10 days) with AM404. Pretreatment with cannabinoid antagonists showed that only the selective CB2 receptor antagonist AM630 was able to block AM404-induced MeCP2 and HDAC2 expression in the dorsal striatum and nucleus accumbens core. In the nucleus accumbens shell, both CB1 and CB2 receptors antagonists blocked the AM404 effect. We further treated rats directly with the selective CB2 receptor agonist JWH133. This was sufficient to increase MeCP2 and HDAC2 expression in caudate putamen and core subregion of nucleus accumbens. In parallel, we observed decreased acetylated levels of histone H3. Transcripts for MeCP2 and HDAC2 levels were not modified by this treatment. Also, some genes coding proteins from the endocannabinoid system were regulated by the JWH133 treatment. Our findings highlight a novel property of the CB2 receptor, which is to potentially control the transcription of an array of genes in the striatum through the induction of MeCP2 and of one of its co-repressor, HDAC2.

Introduction

Drug addiction is a complex brain disease inducing long-term neuroplasticity and leading to uncontrolled drug intake, compulsive drug seeking, craving for the drug and to a high probability of relapse. Among the neurobiological mechanisms involved in addictive behaviors, epigenetic processes are emerging as crucial effectors of the long-term adaptations produced by drugs of abuse. These mechanisms concern chromatin remodeling and include DNA methylation, as well as acetylation and methylation of histones. In the last decade, such dynamic modifications have been studied in neurons in relationship with neurodevelopmental, psychiatric and neurodegenerative disorders (Cholewa-Waclaw *et al.*, 2016; Nestler *et al.*, 2016; Francelle *et al.*, 2017). In particular, neuroepigenetic mechanisms are involved in adaptations to alcohol, opiate, cocaine and cannabis (for reviews see (Nestler, 2014; Farris *et al.*, 2015; Zwiller, 2015; Pandey *et al.*, 2017; Szutorisz & Hurd, 2018)).

Among abused drugs, cannabis is the most popular illicit substance worldwide. This drug interacts with the endogenous cannabinoid (eCB) system, which plays a role in several cognitive functions including memory, nociception, reward and mood regulation. This system comprises lipid neuromodulators (endocannabinoids), enzymes for their synthesis and degradation, and two well-characterized receptors, the cannabinoid receptors CB1 (CB1R) and CB2 (CB2R). Both receptors belong to the superfamily of G protein–coupled receptors that interact with the Gi/o class of G proteins and share common signaling properties. CB1R are highly expressed in the brain and their role in reward has been widely described (Panagis *et al.*, 2014; Befort, 2015; Zimmer, 2015) while the contribution of CB2R, initially considered as a peripheral receptor, has only recently been proposed. Indeed, CB2R expression has been reported in several brain regions including hippocampus, striatum and thalamus (Wotherspoon *et al.*, 2005; Gong *et al.*, 2006; Onaivi *et al.*, 2006; Li & Kim, 2015; Stempel *et al.*, 2016) as well as in the ventral tegmental area (VTA) (Zhang *et al.*, 2014, 2017). The precise role of CB2R expressed in these various brain regions is not well known, but recent studies have proposed a role for this receptor in addictive responses. Its role in alcohol is rather complex, as a selective blockade of CB2R prevents the development of alcohol preference, while selective activation of CB2R enhances alcohol preference, in mice subjected to chronic mild stress (Ishiguro *et al.*, 2007). Activation with beta caryophyllene CB2 agonist rather decreased alcohol intake and preference mice (Al Mansouri *et al.*, 2014). Using mice deficient for CB2R, others have shown that the absence of the receptor increased voluntary ethanol consumption, preference and motivation to drink ethanol (Ortega-Álvaro *et al.*, 2015). Remarkably, a very recent report showed that selective deletion of CB2R in dopamine-expressing neurons (conditional knockout mice) reduced the rewarding properties of alcohol, and selective activation of CB2R block alcohol- place preference (CPP) in wild-type mice (Liu *et al.*, 2017). These results strongly suggest a role for these receptors in reward responses. Interestingly, CB2 also had a protective effect on deleterious effect of ethanol on cell proliferation as its activation rescued

ethanol-induced impaired neurogenesis (Rivera *et al.*, 2015). CB2 is also involved in nicotine responses as invalidation of CB2R using KO mice or blockade with the AM630 antagonist induced a decrease of nicotine self-administration (Navarrete, Rodríguez-Arias, *et al.*, 2013) and nicotine-CPP (Ignatowska-Jankowska *et al.*, 2013; Navarrete, Rodríguez-Arias, *et al.*, 2013). Decreased nicotine-induced somatic signs of withdrawal were observed both following blockade with the AM630 antagonist or in knockout (KO) mice (Navarrete, Rodríguez-Arias, *et al.*, 2013) while the latter effect was not observed in KO mice on a different genetic background (Ignatowska-Jankowska *et al.*, 2013). Implication of CB2R has also been demonstrated for cocaine responses, with a decrease of reinforcing effect in mice overexpressing CB2R (Aracil-Fernández *et al.*, 2012) or blockade of cocaine-CPP using an agonist selective for CB2 (Ignatowska-Jankowska *et al.*, 2013). Interestingly, no difference between wild-type and CB2R KO mice were observed in cocaine-CPP (Ignatowska-Jankowska *et al.*, 2013). In addition, a decrease of cocaine self-administration and attenuation of cocaine-enhanced extracellular DA, was observed following selective activation of CB2 directly in the nucleus accumbens (NAc) or VTA. These latter effects were blocked by AM630, a selective CB2R antagonist (Xi *et al.*, 2011; Zhang *et al.*, 2014, 2017). Altogether, these results highlight a role for CB2R in reward-related behaviors but whether these effects are directly mediated by CB2 activation is not clear yet.

Among epigenetic mechanisms, alteration of DNA methylation participates to the regulation of gene expression. The methyl-CpG-binding protein MeCP2, a protein binding methylated DNA, elicits the recruitment of histone deacetylases (HDACs), which by removing acetyl groups from key histone residues, promote an inactive chromatin state, resulting in the silencing of downstream genes (Klose *et al.*, 2006). MeCP2 is involved in the functioning of mature neurons by regulating spontaneous neurotransmission and short-term synaptic plasticity (Na & Monteggia, 2011). HDAC class I family members, especially HDAC2, are recognized as major player in cognitive functions, as they cause memory impairment when over-expressed (Akhtar *et al.*, 2009; Guan *et al.*, 2009). DNA methylation has been studied in addiction processes, mostly for cocaine or alcohol use disorders (for reviews, see (Vaillancourt *et al.*, 2017; Zhang & Gelernter, 2017)). We previously reported that psychostimulants like cocaine or nicotine induced the expression of epigenetic factors like MeCP2 and HDAC in several rat brain regions including the NAc (Cassel *et al.*, 2006; Pastor *et al.*, 2011). The present study was therefore designed to examine whether modulating the endocannabinoid system, with a focus on the CB2 cannabinoid receptors, was able to regulate MeCP2 and HDAC2 expression in the mesocorticolimbic system. We also investigated whether CB2R activation may directly modulate transcripts from the eCB system.

Materials and Methods

Subjects

Male Wistar rats (Janvier, France) weighting 220–270 g at the beginning of the tests were habituated for two weeks to housing conditions in a temperature and humidity controlled environment with a 12 h light/dark cycle (lights on at 07:00 AM). Rats were group housed in standard cages with *ad libitum* access to food and water. All experimental procedures were performed according to the European Union laws for animal studies and approved by the institutional ethics committee CREMEAS (Comité d'Éthique pour l'Expérimentation Animale de Strasbourg, France) (67-165 to J.Z and 67-300 KB at time of experiment running). All efforts were made to minimize animal suffering and to reduce the number of animals used.

Drugs

Drugs were purchased from Tocris Bioscience (UK) and freshly prepared before each experiment. AM404 (N-(4-hydroxyphenyl)-5Z,8Z,11Z,14Z-eicosatetraenamide)(5 mg/kg), AM251 (N-(piperidiyl)-5-(4-iodonophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide) (3 mg/kg) and AM630 (6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methadone) (3 mg/kg) were dissolved in a NaCl 0.9% solution containing, 10% dimethyl sulfoxide (DMSO) and 1% Tween-20. JWH133 ((6a*R*, 10a*R*)-3-(1,1-dimethylbutyl)-6a, 7, 10, 10a-tetrahydro-6, 6, 9-trimethyl-6*H*-dibenso[*b,d*]pyran) (1 or 3 mg/kg) was dissolved in Tocrisolve^{TM} 100. The corresponding vehicle solutions were used for controls. Solutions were administered intraperitoneally (i.p.) in a 1 ml/kg volume.

Immunohistochemistry

Animals were given an overdose of pentobarbital (500 mg/kg, i.p.) at 15 h following either acute drug injection or the last injection of a series of ten (one/day) in the case of chronic treatment. They were then transcardially perfused with 100 ml saline followed by 2% paraformaldehyde in phosphate-buffered saline (PBS; 0.1 M; pH 7.2; 250 ml). Brains were removed, kept overnight at 4°C in 15% sucrose, frozen in isopentane at -40°C, and stored at -80°C. Immunohistochemistry was performed as described previously {Cassel, 2006 #28}. Briefly, coronal brain sections (20 µm thick) were incubated overnight with the following primary polyclonal antibodies, obtained from Millipore (MA, USA): anti-MeCP2 (1:150 dilution); anti-HDAC2 (1:200 dilution); anti-K9/K13Ac-H3 (1:200 dilution); or anti-K5/K8/K12/K16Ac-H4 (1:2000 dilution). Sections were then successively incubated with biotinylated donkey anti-rabbit IgG (1:500 dilution, Vector Laboratories, CA) and with an avidin–biotin peroxidase complex (Vectastain ABC kit, Vector Laboratories, CA). Staining was revealed with the chromagen 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2. Sections were then incubated in a 5.0 µM bisbenzimide (Hoechst 33258; Sigma-Aldrich, MO, USA) solution to label nuclei and the slides were coverslipped with Mowiol (Calbiochem, MA, USA). Staining was observed under a fluorescent Leitz DM RB binocular microscope (Leica Microsystems, Wetzlar,

Germany). Two photomicrographs of the same field were taken with an Axiocam camera (Carl Zeiss, Iena, Germany); one was used to count the number of nuclei stained with Hoechst 33258, representing the total number of cells and the other to count the number of immunoreactive cells for a given antigen. Counting was achieved using the plugin Cell Counter tool of ImageJ 1.43 software (NIH, MA). The percentage of immunoreactive cells was calculated from counts by an investigator blind to the experimental conditions. For each measure, 6 counts were performed on 4 sections from each rat. Expression was estimated in the cingulate cortex (CgCx), in the nucleus accumbens core and shell (NAcCo and NAcSh, respectively), in the dorsal striatum or caudate putamen (CPu) and in VTA.

Immunoblotting

Animals were given an overdose of pentobarbital (500 mg/kg, i.p.) at 2h (acute and chronic) or 15h (acute) following JWH133 administrations (3 mg/kg or otherwise indicated in text). They were then decapitated and their brain removed, rinsed in cold 1X PBS (phosphate-buffered saline solution, Sigma) and 1-mm thick slices were cut with a stainless steel coronal brain matrix chilled on ice (Harvard apparatus, Holliston, MA, USA). Dorsal striatum (CPu) was microdissected bilaterally using a 3-mm diameter tissue corer according to the rat brain stereotaxic atlas (Paxinos & Watson, 2007). Samples were then homogenized as previously described in 20 mM HEPES buffer, pH 7.9, containing 0.1M KCl, 0.2 mM ethylene-diaminetetraacetic acid, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride and 20% glycerol (Tesone-Coelho *et al.*, 2015). The homogenate was centrifuged at 1000g for 20 min at 4°C and the pellet was resuspended in a Tris buffer (10 mM Tris-base pH 7.9, 0.1mM EDTA, 0.1mM DTT). Identical amount of proteins (15 µg) were separated on a polyacrylamide gel 4-15% and proteins were transferred onto a PVDF membrane (Biorad, France) in Tris-glycine buffer (25 mM Tris-base pH8.3, 192 mM glycine, 1% SDS). Resulting blots were then blocked in PBS-I-block (Tropix, Applied Biosystems), 0.1% Tween 20 buffer for 1h. An overnight incubation at room temperature with the following primary polyclonal antibodies was processed (anti-MeCP2 antibody 1:2000 (Millipore, 07-013); anti HDAC2 antibody 1:2000 (Abcam, ab32117); anti-actin antibody 1:3000 (Sigma Aldrich, A2066). Blots were washed and then incubated in biotinylated secondary goat anti-body (1:50 000) for 1h at room temperature (Jackson ImmunoResearch Laboratories, Inc). Antibody binding was revealed by chemiluminescence (ECL Prime, GE healthcare, Piscataway, NJ, USA) detected using the ChemiDoc Imager (Biorad, France).

Quantitative real-time PCR

Microdissection was processed as for immunoblot samples and samples were immediately frozen on dry ice and kept at -80°C. Samples (n=5-10 rats/group) were processed to extract total RNA using TRI Reagent (Molecular Research center Inc, Cincinnati OH, USA) according to manufacturer's instructions. The quality and quantity of RNA was measured with a NanoVue™

(GE healthcare) spectrophotometer. Reverse transcription to obtain cDNA was performed on 1 µg of total RNA in a 20 µL final volume, with iScript (iScript™ cDNA Synthesis Kit, Biorad, France). Real-time PCR was performed using a CFX96 Touch™ apparatus (Biorad, France) and Sso Advanced™ Universal SYBR Green supermix (Biorad, France) in a final volume of 15 µl. Thermal cycling parameters were 30 sec at 95°C followed by 40 amplification cycles of 5 sec at 95°C and 45 sec at 60°C. Primer sequences for all tested genes are given in **Table 1**. Expression levels were normalized to *rplp0* housekeeping gene levels and compared between controls and treated samples using the 2-ΔΔCt method (Livak & Schmittgen, 2001).

Statistical analysis

All results are expressed as mean ± sem. Data from Western blots were analyzed using one-way ANOVA (treatment) and student t-test. Immunohistochemistry data for each structure and epigenetic factor were analyzed using two-way ANOVA (treatments and period). Transcripts analysis in CPu was also performed using two-way ANOVA (treatment and period). Student-Newman-Keuls *post hoc* test was applied when required. Data obtained in the IHC experiment in which JWH133 was injected were analyzed using the Student's *t* test. Significance was set at $p \leq 0.05$.

Results

Acute inhibition of the anandamide transporter increases MeCP2 and HDAC2 expression

To test whether an acute or chronic increase in synaptic anandamide (AEA), an endocannabinoid ligand, was able to modify the expression of MeCP2 and HDAC2, four experimental groups were constituted (n=4/group). Two groups were injected with either vehicle or with 5 mg/kg of the inhibitor of the AEA transporter, AM404. The two other groups received a daily treatment with vehicle or AM404 at the same dosage for 10 days. **Figure 1A** shows the quantitative analysis of cells expressing MeCP2 and HDAC2 in the CgCx, CPu, core and shell subregions of NAc and VTA of rats exposed to AM404. The acute treatment appeared to increase MeCP2 expression throughout the striatum; statistical analysis however showed a tendency in the CPu (interaction between treatment vs period: $F(1, 11) = 4.717$; $p = 0.052$). The acute treatment with AM404 was found to significantly increase HDAC2 expression with similar amplitude in CPu, NACSh and VTA and close to significant in CxCG and NAcCo (CgCx: $F(1, 10)=6.285$, $p=0.031$; CPu: $F(1, 10)=8.002$, $p=0.017$; NAcCo: $F(1, 10)=3.292$, $p=0.099$; NacSh: $F(1, 10)=3.486$, $p=0.091$; VTA: $F(1, 10)=8.684$, $p=0.014$). Surprisingly, no significant differences in the expression of either MeCP2 or HDAC2 were observed when AM404 was injected repeatedly during 10 days **(Fig. 1A).** **Figure 1B** illustrates these results, with the corresponding MeCP2 and HDAC2 immunostaining in the CPu of rats acutely injected

with AM404. Both MeCP2 and HDAC2 immunoreactivities were exclusively found in cell nuclei, as expected for proteins that bind DNA or histones. The number of cells expressing MeCP2 and HDAC2 was increased in the CPu of rats acutely treated with AM404 when compared with the control group. Considering the chronic AM404 treatment, no significant difference was observed in the number of MeCP2- and HDAC2-expressing cells.

CB2 receptor antagonist blocks AM404-induced MeCP2 and HDAC2 protein expression

We next investigated whether the MeCP2/HDAC2 induction observed in response to the increased availability of AEA was due to the stimulation of CB1R and/or CB2R. We therefore conducted a pilot experiment and treated the rats 30 min before the injection of AM404 or vehicle with either the CB1R antagonist AM251 (3 mg/kg), the CB2R antagonist AM630 (3 mg/kg), or vehicle solution (n=2 to 3/group). Since the AM404 effect was mostly observed in the CPu and NAc, particular attention was further given to the striatum. No effect on the protein level of both epigenetic factors was found in response to either antagonist injected alone, when compared to the control group (**Sup Fig1A**). Results from this pilot experiment indicate that AM404 induced both MeCP2 and HDAC2 levels, an effect that was not blocked with AM251, except in NacSh for HDAC2. In contrast, we found that throughout the striatum, pre-treatment with the CB2R antagonist AM630 completely reversed the increase in HDAC2 expression elicited by AM404 treatment. Data for HDAC2 immunostaining in the dorsal striatum are illustrated in (**Sup Fig 1B**). Effect of the antagonist on MeCP2 levels is less clear.

Activation of CB2 receptor regulates MeCP2 and HDAC2 protein expression as well as histone acetylation status in the dorsal striatum

Since HDAC2-induced expression was blocked by a CB2R antagonist in our pilot experiment, we investigated the effect on their expression following a direct CB2R activation. We used JWH133, a selective ligand for CB2R, which lack in vivo functional pharmacological effect indicative of CB1 activity (Soethoudt *et al.*, 2017). Two groups of rats were therefore treated acutely with 3 mg/kg JWH133 or vehicle. Quantitative analysis of cells expressing the epigenetic factors in the striatum is shown in **Figure 2A**. JWH133 was found to increase MeCP2 expression throughout the striatum; however statistical significance was only reached in the CPu, with an increase of about 50% (CPu: p=0.0001). HDAC2 expression was induced by JWH133 in the CPu and in the NAcCo, while no effect was noticed in the NAcSh (CPu: p=0.005; NAcCo: p=0.017; NAcSh: p=0.696). This observation was further confirmed by the measurement of the acetylation levels of histones. We found a decreased level of Ac-H3 in all three striatal regions (Ac-H3 in CPu: p=0.0016; NAcCo: p=0.0006; NAcSh: p=0.029). The acetylation status of histone H3 was decreased in the NAcSh,

even if the increase in MeCP2 and HDAC2 expression did not reach significance in this striatal subregion (see above). No such decrease was observed concerning the acetylation level of histone H4 (AC-H4 in CPu: p=0.557; NAcCo: p=0.219; NAcSh: p=0.812). Immunostaining results illustrated in **Figure 2B** show that the number of immunoreactive MeCP2 and HDAC2 cells in the CPu was clearly enhanced upon a single JWH133 injection. A great amount of cells from the dorsal striatum expressed acetylated forms of histone H3 on Lys9 and -13 and of histone H4 on Lys5, -8, -12 and -16. Interestingly, only the number of cells expressing the acetylated form of histone H3 was decreased by the JWH133 injection. The data show that acute stimulation of the CB2R is sufficient to induce the MeCP2/HDAC2 complex.

A separate cohort of rats was treated acutely with vehicle, 1, or 3 mg/kg JWH133 (n=5/ group) and dorsal striatum samples were processed for Western blot analysis. The data confirm the immunohistochemistry results, with a significant increase of protein levels at the two tested doses of the CB2 agonist for both MeCP2 and HDAC2 (MECP2 : $F(2, 13)=9.217$, p=0.0032 ; JWH1mg p=0.001 and JWH3mg p=0.032) (HDAC2 : $F(2,12)=21.375$, p=0.00011; JWH1mg p=0.00033 and JWH3mg p=0.0086) (**Figure 3A**).

Activation of CB2 receptor does not modify MeCP2 and HDAC2 transcripts in the dorsal striatum

To further characterize the effect of CB2 activation on these epigenetic factors, we examined their mRNA levels in the dorsal striatum in the same condition, 15h following acute administration of JWH133 (3 mg/kg). No significant difference was observed for MeCP2 or HDAC2 transcript levels compared to control group (MECP2: p=0.236; HDAC2: p=0.534; n=5-6/group) (**Figure 3B**). As expression of epigenetic factors is a dynamic process {Host, 2011 #63}, we also examined whether a shorter kinetic following the drug administration could allow to detect any changes in RNA expression. A group of rats (n=5/group) received an acute dose of JWH133 (3mg/kg) or vehicle and was sacrificed 2 h following the injection. No regulation of MeCP2 and HDAC2 mRNA expression could be observed in these conditions (MeCP2: p=0.747; HDAC2: p=0.51). Similarly, a chronic treatment for 10 days (JWH133, 3 mg/kg, 1 injection/day, n=5/group) did not modify MeCP2 or HDAC2 mRNA expression in these conditions (MECP2: p=0.559; HDAC2: p=0.556).

Activation of CB2 receptor modulates the expression of endocannabinoid-related genes

We further examined the impact of CB2 activation (JWH133, 3mg/kg, acute and chronic) on the expression of several genes belonging to the endocannabinoid system. We measured RNA levels in the dorsal striatum for the two cannabinoid receptors CB1 (*cnr1*) and CB2 (*cnr2*) and CRIP1A, a cannabinoid interacting protein (*cnrip*), as well as for the enzymes that degrade the endocannabinoids (*Mgll* and *FAAH*) (**Figure 3B**). Neither *cnr1* nor *cnr2* were regulated by a single

injection of JWH133. Interestingly, *cnrip* was significantly increased by 1.6 fold only in the acute condition after 15h (p=0.031). A trend to significant increase was also observed for cnr1 following JWH133 chronic injection (p=0.052). Finally, a tendency to down-regulation of both enzymes for degradation of endocannabinoids 15h following acute CB2 activation (FAAH: p=0.08 and *Mgll*: p=0.09) was observed while an up-regulation was detected at the earlier time-point (2h) for Mgll (*Mgll*: p=0.0004), illustrating a transient dynamic regulation for these two transcripts. No difference in expression levels for these transcripts was observed in the chronic treatment.

Discussion

In this report, we show that stimulation of CB2R is sufficient to induce the epigenetic factors MeCP2 and HDAC2 in striatum. This is revealed by the observation that (i) the selective CB2R agonist JWH133 increased the expression of both factors at the protein level; (ii) the blockade of AM404-induced HDAC2 expression by the selective CB2R antagonist AM630. This finding was particularly noticeable in rat striatum. A single JWH133 administration also slightly modulated some endocannabinoid-related gene transcripts.

Transient effect of AM404 on epigenetic factors.

The effect of AM404 was observed in response to an acute injection but vanished after ten daily injections. The induction of both epigenetic factors was also observed following a single activation of CB2R. This probably results from the desensitization of some elements of the cascade leading to MeCP2 expression. It might well be the receptor itself that was desensitized; unfortunately, little is known about CB2R desensitization. There is some evidence from heterologous expression systems that CB2R can undergo internalization following JWH133 stimulation. Also, such a treatment promotes membrane recruitment of β-arrestin2 (Atwood *et al.*, 2012). A recent study revealed potential biased-signaling following CB2 activation in vitro (Soethoudt *et al.*, 2017) which may also participate to this transient physiological effect of JWH133. It is noteworthy that the opposite observation was found when psychostimulants were injected to rats; we previously observed an induction of RNA and protein MeCP2 levels to a much higher level in the striatum, the frontal cortex and the dentate gyrus, when cocaine was injected daily for ten days than after an acute injection (Cassel *et al.*, 2006). Moreover, after a similar chronic protocol, binding of MeCP2 to the PP1Cβ promotor was increased (Pol Bodetto *et al.*, 2013). This opposite modulatory effect is probably due to the fact that cocaine binds primarily monoamine transporters, while in the present study we activated G-protein coupled receptors, whose long-term activations are known to produce adaptations at both system and cellular levels.

A selective effect from CB2 activation.

Even if AEA displays lower relative intrinsic activity for CB2R than for CB1R (Di Marzo *et al.*, 2002), elevated concentrations of AEA in the synaptic cleft may also activate CB2R. Administration of AM404 is known to significantly increase the amount of AEA, allowing the stimulation of both receptors. We found that CB1R blockade by AM251 did not impair the AM404-induced expression of MeCP2 and HDAC2 in the CPu. Our data showing a blockade of MeCP2 and HDAC2 expression by the CB2R antagonist AM630 highlights a CB2R-mediated effect. Altogether, the data indicate that the epigenetic parameters are under the control of CB2R stimulation in the CPu and NAcCo. The situation was different in the shell subregion of NAc, where, both AM251 and AM630 pretreatment were able to block AM404-induced HDAC2 expression. In this specific substructure, distinct receptor expression or potential CB1R-CB2R interaction may explain the implication of both receptors in this adaptation. One study has demonstrated that the two receptors can form heterodimers, which may result in a cross-antagonism phenomenon (Callén *et al.*, 2012). Also, a specialized role for the shell subregion of NAc versus the core region at the molecular level may be involved in this joint effect of cannabinoid antagonists on HDAC2 expression (Salgado & Kaplitt, 2015). Besides the classical cannabinoid receptors, there is growing evidence that the TRPV1 channel (transient receptor potential cation channel subfamily V member 1/ vanniloid receptor 1) participates to the endocannabinoid signaling. TRPV1 is a polymodal transient receptor potential vanilloid channel expressed in post-synaptic neurons, in which it regulates synaptic function (Pertwee *et al.*, 2010; Castillo *et al.*, 2012). AEA binds TRPV1 (Di Marzo *et al.*, 2002) and AM404 is considered as an agonist of this channel {Yue, 2004 #45}. Whether the effect of AM404 on the induction of the epigenetic parameters occurred through the TRPV1 channel can nevertheless be ruled out since the effect was mimicked by the very selective CB2R agonist JWH133 and blocked by its antagonist AM630. The only TRP channels that is activated by JWH133 is TRPA1, but with a rather low potency. Indeed, JWH133 has recently been described as a gold standard selective agonist for CB2 (Soethoudt *et al.*, 2017).

Gene regulation following CB2R activation

We could not detect any changes in MeCP2 or HDAC2 mRNA expression in our conditions in the dorsal striatum. This is in line with our previous findings with no detectable changes of MeCP2 transcript levels while the protein was induced following cocaine-self administration in rats (Pol Bodetto *et al.*, 2014). Also, it is possible that CB2R activation regulates MeCP2 and HDAC2 protein levels as a direct activation of translational process or inhibition of their degradation, as these changes may be transient and dynamic (Host *et al.*, 2011). Interestingly, CB2R activation seem to upregulate the expression of CB1R, but only following a chronic treatment, which may reflect a long-term adaptation in the striatum. Previous studies have shown differential regulation at the level of RNA or protein for CB1R following repeated treatment with Δ^9-tetrahydrocannabinol (Δ^9-THC). This compound is the primary psychoactive compound of cannabis and acts at both CB1R and CB2R as partial agonist. In rat striatum, CB1 mRNA was increased while decreased levels of

protein were measured following repeated Δ⁹-THC treatment (Romero *et al.*, 1997). Also, regulations are region-dependent following cannabinoid self-administration paradigm (Fattore *et al.*, 2007). In addition, chronic Δ⁹-THC treatment induced CB1 desensitization and downregulation in several brain regions, with the least one affected being the striatum (Lazenka *et al.*, 2013). These results indicate a potential primary action of Δ⁹-THC on the receptor protein itself with a possible compensatory effect at the RNA level. As Δ⁹-THC acts also at CB2R, taken together with our results, these data suggest an implication of CB2R in CB1R regulation. Similarly, psychostimulants like cocaine can differentially regulate CB1 mRNA expression depending on the brain regions and on the duration of treatment. Expression is lowered by an acute cocaine injection in mouse prefrontal cortex and cerebellum while an increase is observed in mouse hippocampus (Blanco *et al.*, 2014, 2016; Palomino, Pavón, *et al.*, 2014) as well as following a chronic treatment (Blanco *et al.*, 2016) but no data are available for the striatum. Several studies have examined CB1R regulation following opiate drugs and revealed up-regulation in the striatum at the level of mRNA and/or protein (Rubino *et al.*, 1997; González, Grazia Cascio, *et al.*, 2002; Fattore *et al.*, 2007; Zhang, Wang, *et al.*, 2016). Altogether, these data demonstrate that several drugs of abuse can modulate CB1R and that selective CB2R activation may also participate to these adaptations. In our conditions both enzymes of degradation were down regulated (tendency) by an acute administration of the CB2 agonist (15h post treatment), and *Mgll* was significantly increased at the earlier time point (2h), highlighting a dynamic process. These enzymes have been shown to be regulated by cocaine, with an increase of FAAH in mouse prefrontal cortex and hippocampus following a chronic treatment (Blanco *et al.*, 2014, 2016). Such regulations may participate to adaptations of the endogenous tone of eCB and could also participate to long-term CB1 regulation. Although we acknowledge that we have not assessed eCB tone, further studies examining the endogenous cannabinoids ligand levels following a direct CB2R activation would help to clarify mechanisms involved in this adaptation.

Finally, we observed an increase of *cnrip* transcripts following acute activation of CB2R. This gene encodes a recently identified protein that interacts with the C-terminal tail of CB1R (CRIP1a) and regulates its neuronal activity (Niehaus *et al.*, 2007; Smith *et al.*, 2015) and endocytosis (Blume *et al.*, 2017; Mascia *et al.*, 2017). Alternative splicing of cnrip gene can also produce CRIP1B, potentially also interacting with CB1R as suggested by in silico studies (Singh *et al.*, 2017). Whether CRIP1a or b also modulate CB2R function is not yet known. Moreover, lack of specific antibodies for CRIP1 proteins is a limiting factor to further characterize these regulations at the protein levels. Whether CB2R activation directly interacts with CRIP1 or regulates its expression to modulate CB1 activity need further characterization. Interestingly, we have previously shown that self-administration of cocaine induced an increase of DNA methylation in the promoter region of *cnrip* gene, while no regulation of hydroxymethylation was observed in this gene (Fonteneau *et al.*, 2017). Future studies are likely to focus on the epigenetic control of these

transcriptional adaptations to explore whether these genes (*cnr1, Faah, Mgll, and cnrip*) are directly targeted by MeCP2 complex. Previous studies showing higher rate of promoter methylation of CB1R in peripheral blood of patients dependent to cannabis (Rotter *et al.*, 2013) are indicative of the existence of such regulations.

Mechanisms of regulation

Blockade of cannabinoid transporter by AM404 was found to be sufficient to induce the epigenetic markers. This implies a tonic eCB signaling occurring in the absence of receptor stimulation. On the other hand, the amplitude of eCB tone was not sufficient to be revealed by receptors antagonists, given that CB1R and CB2R antagonists produced no effect when injected alone. Our data are in agreement with a former study showing that tonic eCB signaling is best identified by inhibiting cannabinoid uptake and it is not revealed by CB1R blockade (Wilson & Nicoll, 2001). The mechanism whereby CB2R stimulation triggers the expression of MeCP2 and HDAC2 represents a very challenging issue. The neuroprotective effect of eCBs has been shown to comprise the PI3K/Akt transduction pathway triggered by CB2R stimulation (Molina-Holgado *et al.*, 2002). Mutations within the MeCP2 gene are related with Rett syndrome, a neurodevelopmental disease (Amir *et al.*, 1999). A dysregulation in Akt signaling has been reported in an isogenic human embryonic stem cell model (Li, Wang, *et al.*, 2013)and an animal model of Rett syndrome (Ricciardi *et al.*, 2011) suggesting that MeCP2 is somehow related to the Akt pathway. It is conceivable, for instance, that CB2R stimulation triggers MeCP2 expression through Akt pathway. MeCP2 has classically been described as a global transcriptional repressor (Nan *et al.*, 1997). Transcriptional profiling of MeCP2-null mice brains, however, displayed only subtle changes in gene expression (Tudor *et al.*, 2002). Collectively, microarray data performed with MeCP2 mutant mice or Rett syndrome patients indicate that MeCP2 is not strictly involved in global transcription repression. It rather represses only a limited number of target genes with local expression changes confined to specific brain structures. Transcriptional repression is best characterized via its interaction with HDAC/Sin3 complex (Nan *et al.*, 1997; Klose *et al.*, 2006). By removing acetyl groups from key histone residues, HDACs then promote an inactive state, resulting in the silencing of downstream genes. MeCP2 and class I HDACs, which are among the HDACs recruited by MeCP2, have been shown to be involved in the synapse maturation and function (Nan *et al.*, 1998; Na & Monteggia, 2011). Given the few characterized target-genes of the MeCP2/HDAC2 complex, it is difficult at this point to propose neurobiological mechanisms or behaviors controlled by CB2R stimulation that might involve the epigenetic markers. Since MeCP2 is only induced in response to acute cannabinoid signaling, it is unlikely that the effect is involved in the inhibition by CB2R agonists of cocaine self-administration (Xi *et al.*, 2011; Zhang *et al.*, 2014) or by CB2R antagonists of nicotine self-administration (Navarrete *et al.*, 2018). On the other hand, this cannot be completely ruled out since intra-NAc MeCP2 overexpression was shown to decrease amphetamine-CPP (Deng *et al.*, 2010). Overall, together with data showing an emerging

role for CB2R in rewarding properties of drugs of abuse, this project highlights a potential contribution of epigenetic processes induced by direct CB2 activation. Additional data are needed to ascertain a role for MeCP2/HDAC complex in the effects of CB2R stimulation.

Acknowledgments

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References

The references are available at the end of the thesis manuscript.

Figures

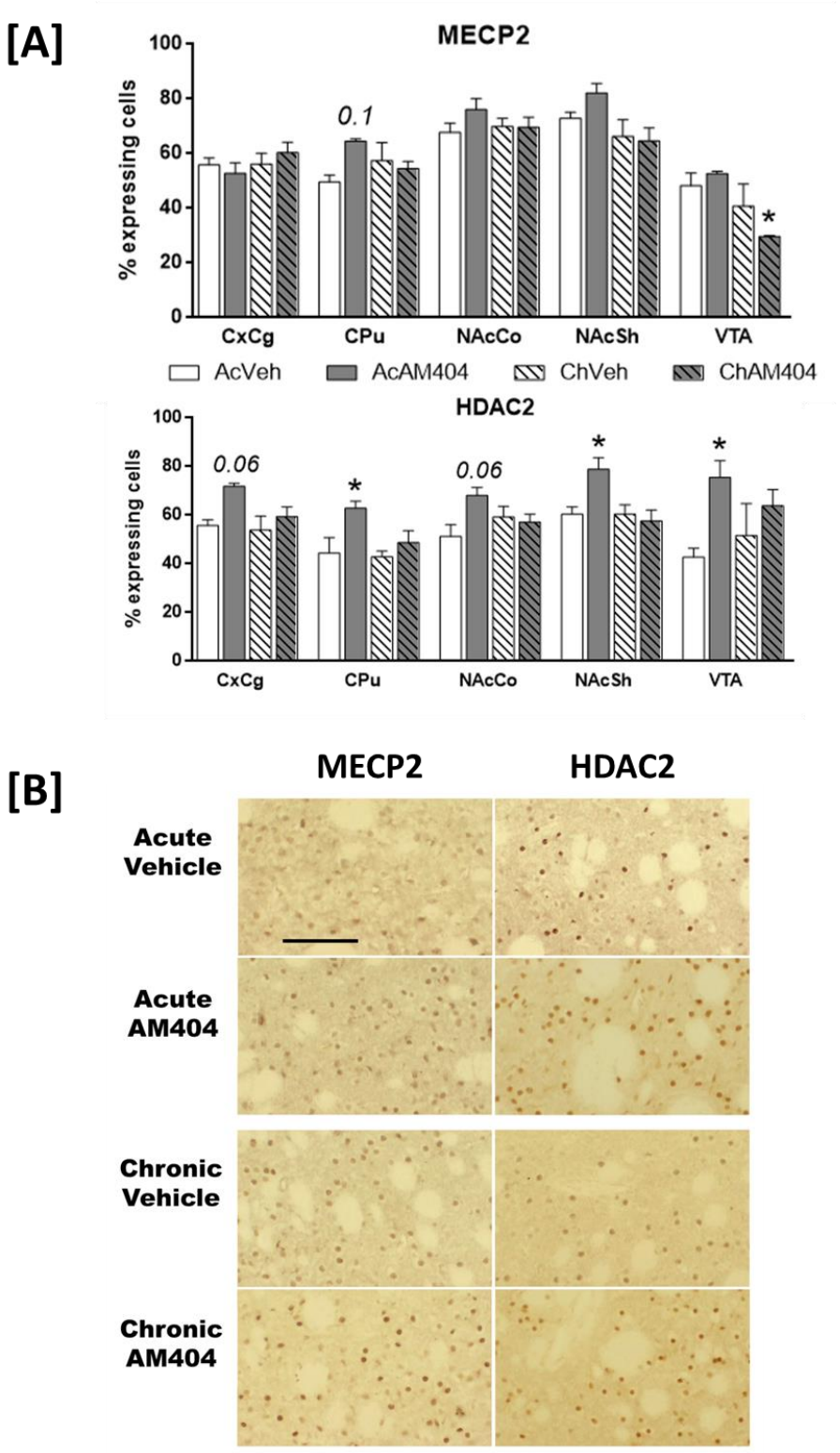


FIG1

Figure 1. Effect of acute and chronic treatment with AM404, an inhibitor of the anandamide transporter, on the expression of MeCP2 and HDAC2. (A) Cells expressing MeCP2 and HDAC2 were quantified 15 h after a single i.p. injection or a daily treatment for 10 days with 5 mg/kg

AM404 or vehicle (n = 4 per group). MeCP2 and HDAC2 expression was analyzed by two-way ANOVA (treatment and period as factors). In animals acutely treated with AM404 (AcAM404), expression of MeCP2 was increased in the caudate putamen (CPu, tendency p=0.1) when compared to the respective control group (AcVeh). The acute treatment with AM404 increased HDAC2 expression in CPu, nucleus accumbens shell (NAcSh) and ventral tegmental area (VTA). A tendency was observed for the cingulate cortex (CgCx, p=0.06) and the nucleus accumbens core (NAcCo, p=0.006). Bars represent means ± S.E.M. *p < 0.05, versus the respective control group (Student-Newman-Keuls *post hoc* test). **(B)** Representative photomicrographs of MeCP2 and HDAC2 immunoreactivity in the CPu of rats submitted to an i.p. acute injection or repeated injections for 10 days with 5 mg/kg AM404 or vehicle. Scale bar applicable to all micrographs, 100 µm.

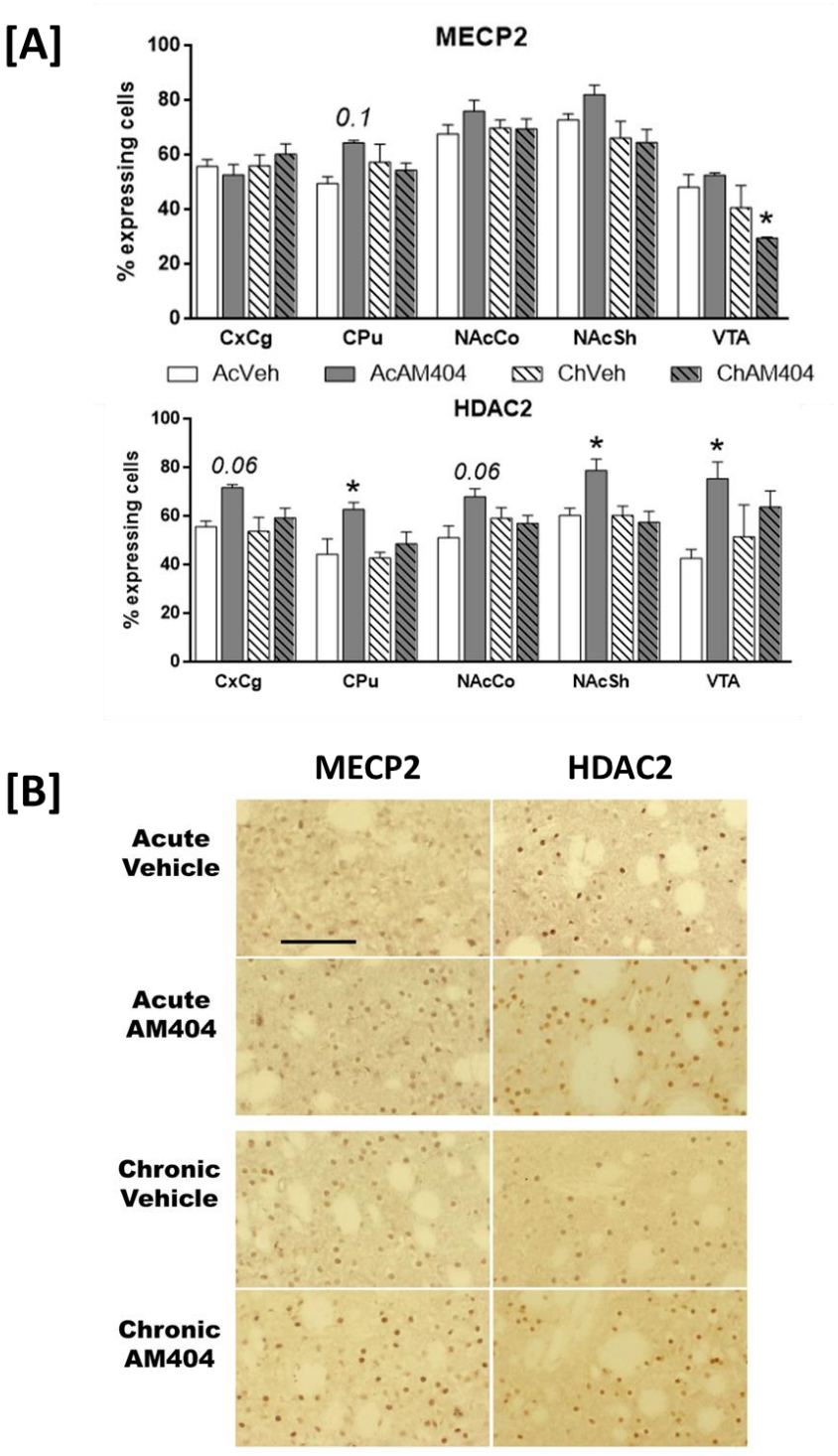


FIG1

Figure 2. Effect of the CB2 agonist, JWH133, on the expression of MeCP2, HDAC2 and on the acetylation levels of histone H3 and H4. (A) The graphs show the quantification of MeCP2, HDAC2, Ac-H3 and Ac-H4 expression in striatum of animals injected i.p. with 3 mg/kg JWH133 (n = 4 per group) and killed 15 h later. In the CPu, JWH133-treated rats displayed higher level of MeCP2 and HDAC2 expression when compared to the control group. Bars represent means ±

S.E.M. *p < 0.05, **p < 0.01, ***p < 0.001 (Student's t test). **(B)** Representative photomicrographs of the immunoreactivity found in the CPu of animals injected i.p. with vehicle or 3 mg/kg JWH133. Scale bar applicable to all micrographs, 100 µm.

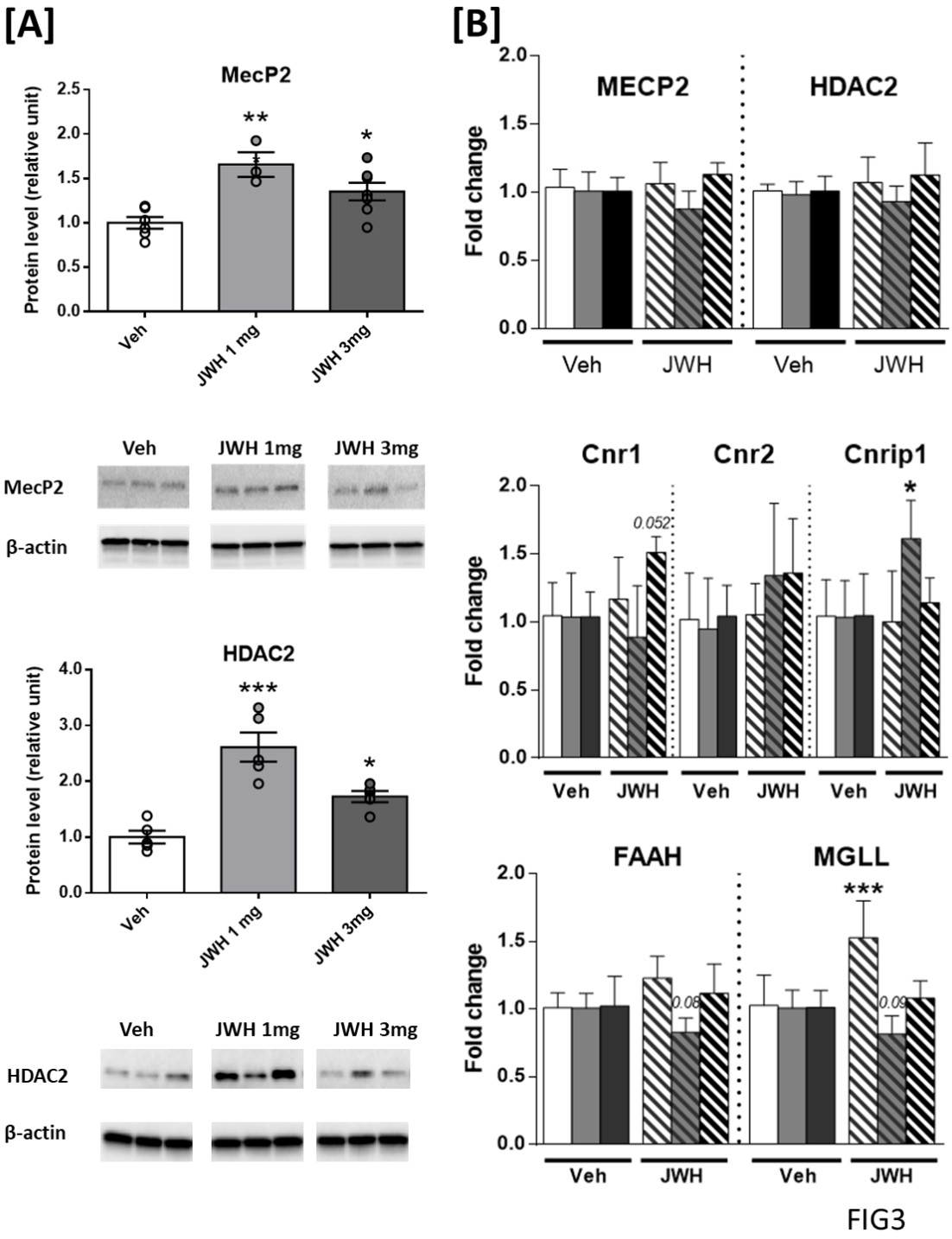


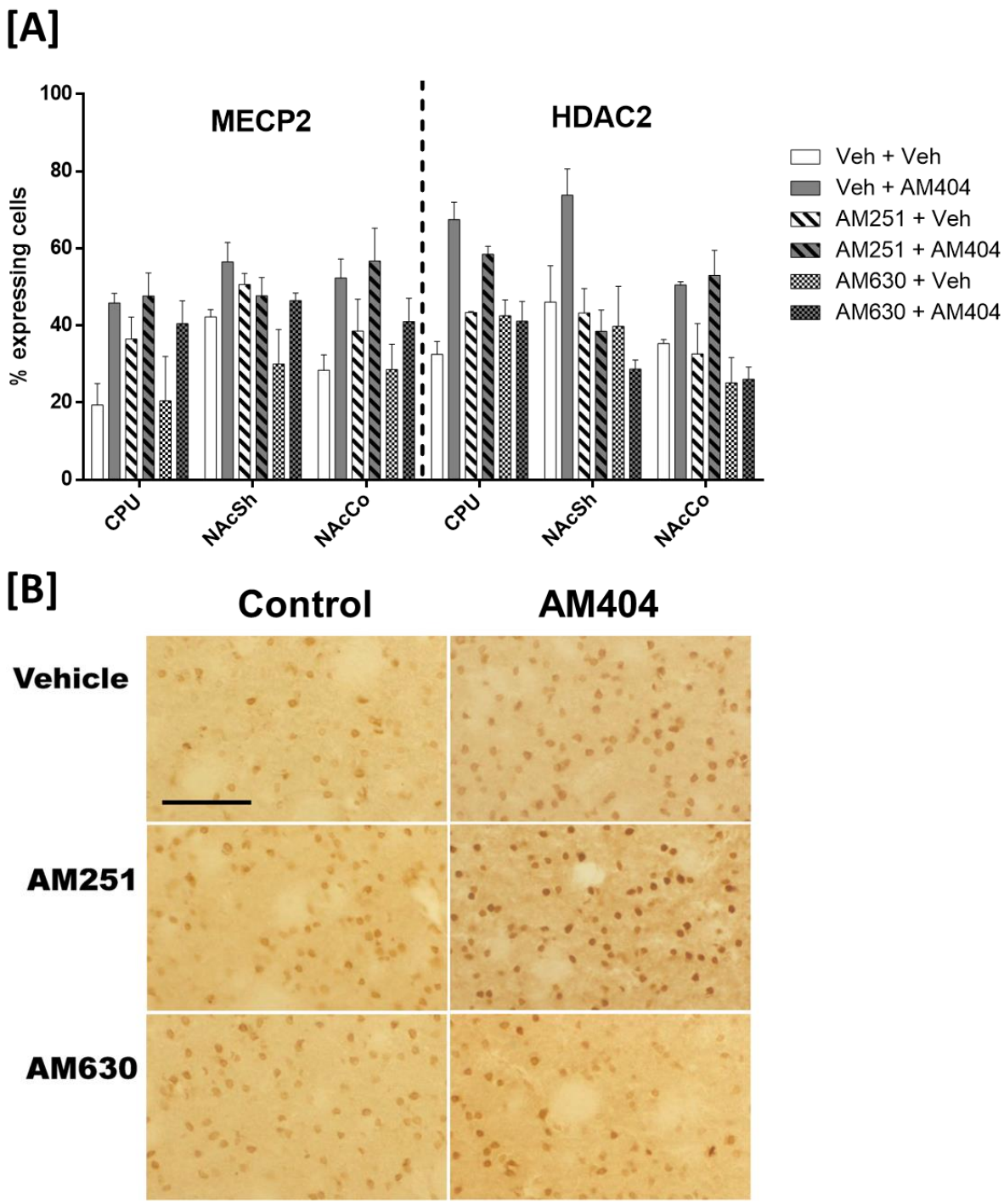
FIG3

Figure 3. Protein and gene regulation following direct CB2R activation. (**A**) Representative immunoblots and quantification of Western blot analysis of MeCP2 and HDAC2 proteins in CPu homogenates following JWH133 administration (1mg/kg, grey and 3 mg/kg, black). Histograms represent the mean+S.E.M (n=3-7/group) with * p<0,5, ** p<0,01, *** p<0,001 vs Veh group. **(B)** Transcripts analysis using qPCR on CPu samples following JWH133 treaments (acute :2h (white and hatched bars) and 15h (grey and hatched bars) following the injection; chronic : 2h following the last injection of ten (black and hatched bars), see methods). * p<0,5, *** p<0,001 vs Veh group.

Table 1: Primer Sequences

Gene name	RefSeq	Forward	Reverse
Hdac2	NM-053447	CCCTCAAACATGACAAACCA	TGTCAGGGTCTTCTCCATCC
MecP2	NM-022673	AAGTCTGGTCGCTCTGCTG	TCTCCCAGTTACAGTGAAGTC
Cnr1	NM_012784	TCTGCTTGCGATCATGGTGT	AGATGATGGGGTTCACGGTG
Cnr2 var 1/2/3	NM_001164143	AATGGCGGCTTGGAGTTCAA	TAGAGCACAGCCACGTTCTC
Cnrip	NM_001014232.1	TTCCCGCATCTCTCTTGTCT	GTCCCGTTTACCGCTGTTTA
FAAH	NM_024132.3	CCCCAGAGGCTGTGTTCTTT	GTCAGATAGGAGGTCACGCA
Mgll	NM_138502.2	TCACGTGCTGCAACAAATCT	GTTGAAGAGGCTGGACATGC
Rplp0/36b4	NM_022402	CTGCCCGAGCCGGTGCCATC	TTCAATGGTACCTCTGGAG
Beta-Actin	NM_031144	GTCAGGTCATCACTATCGGC	CCACCAATCCACACAGAGTA

Primer sequences used for real-time qPCR are indicated with gene names and GenBank/NCBI accession numbers (RefSeq).



SUP FIG1

Supplemental Figure 1. Effect of pre-treatment with cannabinoid selective antagonists on the expression of MeCP2 and HDAC2 in the striatum of AM404-treated rats. A pilot experiment was

conducted with n=2 to 3 animals /group. Animals were injected i.p. with vehicle or 3 mg/kg of the antagonist; 30 min later, they received an i.p. injection of vehicle or 5 mg/kg AM404. They were killed 15 h after the second injection. **(A)** AM404 induced expression of both MeCP2 and HDAC2 and this effect was clearly blocked by the CB2 antagonist AM630 for HDAC2 in all striatal structures. Pre-treatment with the CB1 antagonist, AM251, increased HDAC2 expression in the CPu as in the animals treated only with AM404, except in the NAcSh. Effect on MeCP2 are less clear due to a low number of animals per group. Bars represent means ± S.E.M. **(B)** Representative photomicrographs of HDAC2 immunoreactivity in the CPu of rats injected with vehicle, 3 mg/kg AM251 or 3 mg/kg AM630 30 min before the administration of vehicle or 5 mg/kg AM404. Scale bar applicable to all micrographs, 100 µm.

V. Article 5: LSP29166, a novel orthosteric mGlu4 and mGlu7 receptor agonist, reduces cocaine self-administration under progressive ratio in rats

In the context of the investigation of the impact of a novel orthosteric ligand to reduce cocaine motivation, I participated to this work regarding the gene expression and western blot analysis, for both mGluR4 and mGluR7.

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Running title: Pharmacological activation of mGlu4 and mGlu7 receptor reduces cocaine self-administration

Keywords: operant self-administration, LSP-9166, mGlu4, mGlu7, cocaine, motivation

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Abreviations

Amy: amygdala ; AUC: area under curve ; Cocaine-SA: cocaine self-administration ; CPP: conditioned place preference ; DS: dorsal striatum ; HPC: hippocampus; LTP: long-term potentiation; PFC: prefrontal cortex; PR: progressive ratio; NAc: Nucleus Accumbens; VTA: ventral tegmental area

ABSTRACT

Cocaine addiction is a serious health issue in western countries and relapse frequently occurs. Despite the regular increase in cocaine consumption, no specific treatment for cocaine addiction has been found. Critical roles for glutamate neurotransmission in psychostimulant rewarding effect as well as relapse have been suggested and evidence accumulate indicating that targeting mGlu group III receptors could represent a promising strategy to develop therapeutic compounds to treat addiction. In this context, the aim of our study was to evaluate the direct impact of cocaine on mGlu4 and mGlu7 receptor gene expression. In a second step we examined the effect of LSP2-9166, a mGlu4/mGlu7 receptor orthosteric agonist, on cocaine intake. We used an intravenous- self-administration paradigm in male Wistar rats as a reliable model of voluntary drug intake. Voluntary cocaine-intake following a fixed ratio of injections induced an increase of both mGluR4 and mGlu7 receptor transcripts. We evaluated the ability of LSP2-9166 to impact cocaine self-administration under a progressive ratio schedule of reinforcement. We found that this compound inhibits the motivation to obtain the drug although this orthosteric ligand induced a short lasting hypolocomotor effect. Together, our findings support common adaptations to drugs of abuse, including alcohol, morphine and cocaine and highlight that mGlu group III receptors represent new targets for drug relapse.

Highlights

- Cocaine increases mGLU group III receptor transcript expression
- LSP inhibits motivation to self-administer cocaine
- Interest of targeting mGlu group III to treat substance use disorder and relapse

Introduction

Cocaine addiction is a chronic brain disease characterised by a high level of motivation for the drug as well as a high level of relapse (Kalivas *et al.*, 2005). Given the multifaceted health issue that results from this, including mortality and morbidity, research has been conducted to identify the neurobiological mechanisms underlying cocaine rewarding effects and better understand the processes involved in addiction and further propose therapeutic strategies. It is well established that the dopamine (DA) neurotransmission system, particularly the mesolimbic pathway connecting the ventral tegmental area (VTA) to the nucleus accumbens (NAc), is crucial for the motivational component of behavior, measured in rodents by cocaine taking under a progressive ratio schedule of cocaine self-administration (McGregor, 1996; Veeneman *et al.*, 2012). In addition, emerging evidence arised for a major role for glutamate, the main excitatory neurotransmitter in the mammalian brain, in the progressive elaboration of cocaine-seeking behaviors (Kalivas, 2009; Scofield *et al.*, 2016). Even if the dopaminergic regulates glutamate, the glutamatergic neurotransmission also closely modulates the dopaminergic system in the NAc (Girault, 2012) and glutamate levels are modified during relapse (Knackstedt & Kalivas, 2009). Glutamate acts on fast-acting ligand-gated ion channels (ionotropic receptors) and slow-acting G-protein coupled receptors (metabotropic glutamate (mGlu) receptors) and various compounds targeting this system have been developed for the treatment of addiction (D'Souza, 2015). Among the latter receptors, group III (mGlu4, mGlu6, mGlu7 and mGlu8) are mostly pre-synaptic and negatively control glutamate transmission. These mGlu group III receptors have been proposed as targets for several neurodegenerative and neuropsychiatric conditions, including chronic pain, parkinson's and schizophrenia (Crupi *et al.*, 2019; Nicoletti *et al.*, 2019). The mGlu6 receptor being mostly expressed in retina, its role was not examined in addiction. One study investigating the role of mGlu8 showed an inhibition of alcohol self-administration using a selective agonist (Bäckström & Hyytiä, 2005) but the function of this receptor is still not well characterized (Crupi *et al.*, 2019). Recent studies with drugs of abuse have attracted more interest on mGlu4 and mGlu7. Indeed, mGlu4 and mGlu7 are expressed in basal ganglia and structures from the reward circuit, where mGlu7 is often more expressed than mGluR4 (Ohishi *et al.*, 1995; Bradley *et al.*, 1999; Kosinski *et al.*, 1999; Cartmell & Schoepp, 2002; Corti *et al.*, 2002). L-AP4, an agonist of group III mGlu, inhibited Glu and/or GABA release in key-structures of basal ganglia such as striatum and globus pallidus (Valenti *et al.*, 2003; Li *et al.*, 2008; Macinnes & Duty, 2008; Cuomo *et al.*, 2009) and blocked cocaine-induced hyperlocomotion in rats (Mao & Wang, 2000). Interestingly, a recent study showed that a selective mGlu4 agonist, LSP1-2111, reduced sensitization to locomotor effect induced by cocaine or by exposure to a context associated to cocaine (Zaniewska *et al.*, 2014). A selective allosteric agonist of mGlu7, AMN082 (Mitsukawa *et al.*, 2005), reduced cocaine reinforcing properties (Li *et al.*, 2009), cocaine-seeking behavior following extinction (Li *et al.*, 2010) and altered both alcohol-intake and alcohol-conditioned place preference (Bahi, 2012). Side-effects of this mGlu7 compound were observed, with reduction of

sucrose intake (Salling *et al.*, 2008), decreased locomotor activity (Palucha *et al.*, 2007), sleep disturbances (Ahnaou *et al.*, 2016). Anti-depressive and anxyolitic effect were observed and proposed to be linked to a rapid internalization process induced by AMN082 (Cryan *et al.*, 2003; Callaerts-Vegh *et al.*, 2006; Pelkey *et al.*, 2007; Fendt *et al.*, 2013; O'Connor & Cryan, 2013). Finally, AMN082 can be degraded in a metabolite with high affinity for dopamine, norepinephrin and serotonin transporters and could participate to some behavioral consequences of AMN082 administrations (Sukoff Rizzo *et al.*, 2011).

With the design of new orthosteric brain permeant subtype-selective ligands, new investigations were facilitated. Indeed, LSP2-9166 a novel mGluR4/7 orthosteric agonist, (Acher *et al.*, 2012) represents the most powerful orthosteric compound synthezised so far. Remarkably, it was recently shown to inhibit both morphine place preference in mice (Hajasova *et al.*, 2018) and ethanol intake in rats (Lebourgeois *et al.*, 2018). We therefore decided to examine its effect on cocaine motivational properties, as knowledge of molecular and neuroplastic modifications induced by each specific drug of abuse will strengthen the proposed therapeutic interventions that could be beneficial to patients suffering from substance use disorder (SUD). In this study, we investigated whether the pharmacological activation of group III mGlu receptors could modulate cocaine motivation using LSP2-9166 in a preclinical model of cocaine intake in rat. We examined transcript levels for both mGlu4 and mGlu7 in the striatum following voluntary cocaine intake under a low fixed-ratio of reinforcement. We then explored the motivation of rats for cocaine using a progressive ratio schedule of intravenous cocaine self-administration and also evaluated the effect of LSP2-9166 on locomotor activity.

Materials and methods

Animals

Male Wistar rats (Janvier), aged five weeks and weighting 150–174 g upon arrival in our laboratory, were housed in standard home cages (five rats per cage), under an inverted 12 h light/dark cycle (lights on at 7:00 P.M.), with ad libitum water and food. After surgery for catheter implantation into the jugular vein, animals were housed individually so that acoustic, olfactive, and visual social contacts remained possible. They were allowed to recover for 5–7 d before the beginning of behavioral tests which were conducted during the dark period. All procedures involving animal care were conducted in compliance with current laws and policies (Council directive 87848, Service Vétérinaire de la Santé et de la Protection Animales) and were validated by a Comité d'Ethique en Expérimentation Animale (CREMEAS) and authorised by the Ministère de l'Enseignement Supérieur, de la Rechercher et de l'Innovation (ref # 7050-2016093016163350). Two batches of rats were used: 41 rats for the self-administration procedure with LSP treatment and locomotor activity; and 18 rats for the self administration procedure for molecular study.

Pharmacological treatment

Cocaine hydrochloride (Sigma-Aldrich) solution was adjusted with 0.9% NaCl to infuse an intravenous (i.v.) dose of 0.33 or 0.5 mg/kg/injection for self-administration and 1.5 mg/kg (i.v) for locomotor activity. LSP2-9166 is first diluted into distilled water at 20 mg/ml, pH is adjusted to 7.4 and final concentration is set at 2 or 5 mg/ml using Phosphate-Buffer-Saline, PBS. LSP2-9166 (2 or 5 mg/kg, i.v.) or vehicle solution (PBS) was administered 15 min before the beginning of the self-administration session or the locomotor activity test.

Surgery

An i.v. catheterization procedure was performed as described previously (Romieu *et al.*, 2008). Briefly, rats were anesthetized by an intraperitoneal injection (2 ml/kg) of a mixture containing ketamine (Imalgene 1000, 90 mg/kg) and xylazine (Rompun, 10 mg/kg; Centravet) to perform surgical implantation of a chronic indwelling catheter. A 12 cm long tubing (0.3 mm inner diameter x 0.63 mm outer diamter) (SILASTIC; Plastics One) was fitted to a 22 gauge guide cannula (Plastics One) bent at a right angle and then embedded in dental cement on a circular 3 cm mercylene mesh base. A discrete incision was performed onto the right jugular vein and the heparinized catheter was immediately inserted and anchored with suture. Catheters were flushed daily with 150 µL of a solution mixture containing 100 U/mL heparin and 50 mg/mL ampicillin to prevent clotting and infection.

2.2. Cocaine operant self-administration

Apparatus. Studies were conducted in dark operant chambers (30x30x30 cm) located in a sound-attenuated room. A single channel fluid swivel (Instech Laboratories) was mounted on a balanced arm above each chamber. A computer-driven syringe pump (Imetronic, France) activated a 10 mL syringe and pushed fluid into SILASTIC tubing connected to the rat through its externalized 22-g guide cannula. Each chamber was equipped with two 2.5 cm-diameter holes on the same wall, 4 cm above the floor. Holes were selected as active for delivering cocaine and inactive (without programmed consequence) and counterbalanced between right and left position in the various groups of rats. Disruption of an infrared photobeam in each hole (Nose-Poke, NP) was detected using a digital input card (DIO-24; National Instruments) and homemade LabView software (National Instruments). NPs into both holes were recorded. When the required number of NPs into the active hole was reached, a 60 µL cocaine solution was delivered in 2 s. A flashing light stimulus (3Hz), located 20 cm above the active hole, was paired contingently with the delivery of cocaine and persisted during 5 s, followed by a house light illuminating the chamber during a 40 s time-out period.

Drug self-administration procedure. Rats were first submitted to a fixed-ratio (FR) 1 schedule of reinforcement during daily 2 h sessions for 3 d, then submitted to a FR5 schedule of reinforcement for 6 days. No cutoff was applied concerning the number of self-infusions the rat was able to perform during the session. After 9 FR sessions, rats were submitted to a progressive ratio (PR) schedule, in which the NPs required to earn an injection escalated according to the following exponential equation (Richardson & Roberts, 1996), where n is the rank number of successive injections: NP(n) = [5xe0.2n]-5. The PR schedule was performed for 6 consecutive daily sessions followed by pharmacological treatment with LSP2-9166 or vehicle, during 5 daily sessions. Each session lasted for 5 h or until animals did not achieve the ratio for delivery of an injection within 1 h. The breaking point to extinguish self-administration behavior was determined in each animal. For the molecular studies, rats followed a FR1 schedule (cocaine 0.33 mg/kg/injection, 2 h session/day, 10 days) with saline control animals.

Horizontal locomotor activity

Locomotor activity was measured in individual home-cages by means of two infrared light beams perpendicular to the width of the cage, each 4.5 cm above floor level and 28 cm apart

along the length of the cage. Numbers of longitudinal crossings, ie, each time a rat consecutively interrupted the two light beams, were counted and saved in 5-min bins. LSP-9166 or vehicle (PBS) was administered, and locomotor activity was recorded starting 15 min after, for 120 min. Cocaine was then injected to both groups and measures were recorded for 75 min.

Quantitative real-time PCR

All animals were given an overdose of pentobarbital (500 mg/kg, i.p.) and sacrificied 24 hour following the last session of drug injection. Brains were removed, rinsed in cold 1X PBS and 1-mm thick slices were cut with a stainless steel coronal brain matrix chilled on ice (Harvard apparatus, Holliston, MA, USA). Structures of interest were collected by punches according to the rat brain stereotaxic atlas (Paxinos & Watson, 2007) and samples were immediately frozen on dry ice and kept at -80°C. Samples (n=5-10/group) were processed to extract total RNA using Ribozol (VWR, Fontenay-sous-bois, France) according to manufacturer's instructions. RNA quality and quantity was measured with a NanoVue™ (GE healthcare) spectrophotometer. Reverse transcription was performed on 750 ng of total RNA in a 20 µL final volume, with iScript (iScript™ cDNA Synthesis Kit, Biorad, France). Real-time PCR was performed using a CFX96 Touch™ apparatus (Biorad, France) and Sso Advanced™ Universal SYBR Green supermix (Biorad, France) in a final volume of 15 µl. Thermal cycling parameters were 30 sec at 95°C followed by 40 amplification cycles of 5 sec at 95°C and 45 sec at 60°C. Primer sequences are Rplp0 Fw CTGCCCGAGCCGGTGCCATC, Rv TTCAATGGTACCTCTGGAG ; mGlu4, Fw TCCAGGACCAACGGACACTT Rv ACGTGACCATCAGCAGCATG ; mGlu7 Fw AGACACAGAAGGGAACGCCT, Rv TCGGTTCTCATTGGGCCTCT. Expression levels were normalized to *rplp0* housekeeping gene levels and compared between saline and treated samples using the 2-ΔΔCt method (Livak & Schmittgen, 2001).

Statistical analysis

All results are expressed as mean ± sem. Data from qPCR and Western blots were analyzed using unpaired student's t-test (qPCR) or one-way ANOVA (treatment). Data from self-administration experiments and locomotor activity were analyzed using paired t-test or two-way ANOVA (treatments and time) followed by a Sidak's multiple comparisons test. Student-Newman-Keuls *post hoc* test was applied when required. Significance was set at $p \leq 0.05$ (GraphPad Prism Software 7.0, San Diego, CA).

Results

Effect of cocaine on mGlu4 and mGlu7 receptor expression

In a first experiment, we examined whether voluntary cocaine intake would modulate mGlu4 and mGlu7 receptor expressions in reward-related brain structures. Following 10 days of cocaine self-administration under a 2h-daily session under FR1 schedule, rats displayed a stable level of cocaine intake (more than 100 injections/session) and directed more than 90% of the nose pokes (NP) onto the active hole (data not shown). Rats receiving saline did not differentiate between holes (37.8±1.34% of discrimination). Microdissected brain samples were processed to measure gene expression levels by qPCR for mGlu4 and mGlu7 receptors (**Figure 1**). Both transcripts were detectable in all tested areas, with a higher relative expression (> 2-fold) of mGluR7 compared with mGluR4 in Nac, PFC and HPC, as previously described (Ohishi *et al.*, 1995; Bradley *et al.*, 1999; Kosinski *et al.*, 1999; Cartmell & Schoepp, 2002; Corti *et al.*, 2002) (data not shown). Expression of both transcripts were significantly increased in cocaine- compared to saline-treated animals in NAc (mGluR4 p = 0.011; mGluR7 p = 0.0005) and HPC (mGluR4 p = 0.0023; mGluR7 p = 0.0047). A significant decrease was only observed for mGluR4 in Amygdala (mGluR4 p = 0.0024) while no regulations were observed for mGluR7 in amygdala and for both receptors in prefrontal cortex (PFC) and dorsal striatum (DS).

Because mGluR7 has been proposed as the main target of LSP2-9166 (Hajasova *et al.*, 2018), we also examined whether mGluR7 protein levels were altered in NAc using Western Blot analysis but we could not detect any significant changes in our conditions (data not shown).

Effect of LSP-9166 on cocaine motivation

In a second set of experiments, we performed cocaine intravenous self-administration under a FR5 schedule of reinforcement, followed by 6 sessions under a progressive ratio (PR) schedule of reinforcement, in which NPs required for drug delivery followed an exponential ascending equation (see methods). Treatment with LSP2-9166 at 0, 2 or 5 mg/kg i.v. began 15 min before each session, for 5 days. **Figure 2A** presents the number of injections performed by the rats across all sessions in the PR schedule. Because the PR schedule required several self-administration sessions for the rats to display a stable behavior, only the 3 last sessions (sessions 4–6) were taken into consideration for statistical analysis (baseline, BL). Therefore, analysis was performed comparing BL from these sessions with the three last sessions of treatment (T) and mean values are presented (**Figure 2A**). Control rats and rats receiving the compound at 2 mg/kg did not display any behavioral changes in comparison to the training period where no treatment was performed. Their cocaine intake remained stable across sessions. In contrast, daily intravenous administration of LSP2-9166 at 5 mg/kg resulted in a significant reduction in the breaking point, expressed as the number of self-injections reached within a session, during the three last sessions (paired t-test, p=0.0017). The LSP2-9166 at 5 mg/kg reduced the breaking point from 24.9±0.7 to 22.5±0,.6 self-injections. Similar results were obtained when comparing the number of NPs performed by the rats (**Figure 2B**). LSP2-9166 at 5 mg/kg reduced the mean

number of NPs from 5858±811 to 3550±404. It is noteworthy that the NPs achieved into the inactive hole were similar across all groups (data not shown).

Effect of LSP-9166 on locomotor activity

To control for possible effects of the agonist on locomotor activity that would alter the operant behavior in our conditions, we evaluated the effect of LSP2-9166, at the dose of 5 mg/kg, on spontaneous locomotor activity in rats (**Figure 3A**). Locomotor activity progressively decreased the first 20 min in both LSP2-9166-treated and control groups, which may reflect habituation to the set up, while rats are placed in individual cage for mouvement recording. Repeated measures ANOVA revealed a group x time interaction [$F(23,299)=3.82$, $P<0.0001$] When comparing the area under the curve (AUC), we observed a significant decrease in the LSP2-9166-treated group ($z=3.98$; $P<0.0015$), indicating that LSP2-9166 showed a transitory hypolocomotor effect (**Figure 3B**). Sidak's multiple comparisonn test revealed that this effect did not last more than 40 min. No effect was observed on the number of NP in the inactive hole. We further measured cocaine-induced hyperlocomotor activity in both groups, with an iv injection 135 min following LSP2-9166 injection (**Figure 3C)**. Cocaine produced a similar hyperlocomotor activity in both groups, with no significant differences of AUC, indicating no further effect of LSP on cocaine-induced behavior.

Discussion

Our findings highlight that cocaine intake increased mGlu4 and mGlu7 receptor gene expression in both NAc and HPC. For the first time, we show that the orthosteric agonist LSP2-9166 targeting mGlu4 and mGlu7 receptors inhibited motivational and rewarding properties of cocaine, as illustrated by a reduction in the breaking point under a PR schedule.

Since several years, an increasing interest emerged in the addiction field towards mGlu receptors, which are well known to regulate pre- and/or postsynaptic glutamatergic neurotransmission (Cartmell & Schoepp, 2002). Three groups of mGlu receptors exist in the brain. Group I receptors (mGluR1 and mGluR5), mainly localised post-synaptically, were the first to be demonstrated playing a role in drug dependence, and they were proposed as promising therapeutic targets (Mihov & Hasler, 2016). However, mnesic processes were also altered by these compounds (Campbell *et al.*, 2004; Naie & Manahan-Vaughan, 2004). Also, activation of group II mGluR (mGluR2, mGluR3) was shown to reduce both taking and seeking behavior towards distinct drugs of abuse in rodents, through normalization of an altered glutamatergic neurotransmission induced by drug exposure (Knackstedt & Kalivas, 2009). However, mGluR2/3 agonists showed severe side effects, with reduced food-seeking behaviors (Peters & Kalivas, 2006) and a decrease in cognitive performance (Aultman & Moghaddam, 2001; Higgins *et al.*, 2004). Group III mGlu receptors were recently proposed as a new target for treating addiction, with LSP1-2111 (Selvam *et al.*, 2018), an agonist for mGlu4, and AMN802 (Mitsukawa *et al.*, 2005),

acting as the first allosteric mGlu7 agonist. The latter compound was effective to decrease cocaine-induced reinstatement (Li *et al.*, 2010) but also presented side effects. Indeed, AMN082 displayed some behavioral effects in mGlu7-KO mice (Palucha et al., 2007; Ahnaou et al., 2016) and potentiated positive symptoms of psychosis (Wierońska et al., 2012). Recently, the new orthosteric LSP2-9166, examined in our study, was tested for its effect on opiate (Hajasova *et al.*, 2018) or alcohol behavioral responses (Lebourgeois *et al.*, 2018). This ligand efficaciously decreased expression and reinstatement of morphine CPP in mice as well as ethanol consumption, motivation for ethanol and reacquisition of ethanol SA following abstinence in rat, respectively. Our hypothesis that such modulatory effect may be expended to cocaine behavioral response has been tested here and we confirm that this group III mGlu agonist is able to reduce drug consumption.

We also show here, for the first time, that voluntary cocaine intake, under low work requirement (fixed-ratio 1), increased both mGlu4 and mGlu7 transcripts in NAc and HPC, while a decrease was observed only for mGlu4 in Amy. These regulations are region- specific as no modification were detected in PFC or DS. Also, they were observed 24h after the last drug exposure, suggesting that they do not result from short time effect of cocaine. It would be of crucial interest to evaluate whether these regulations are long-lasting, and whether they occur at distinct stages of addiction (withdrawal, seeking events). This would highlight changes that could specifically mediate active drug seeking. Not data are available on the regulation of these receptors by other drugs of abuse, at the level of protein expression. Taken together, our results suggest that cocaine intake can induce an increase in glutamate signaling through the increased mGlu7 and mGlu4 receptor transcripts. This may participate to reduced glutamate release and behavioral limitation of cocaine intake.

We therefore hypothesized that ligand acting at mGlu4/7 receptors could affect cocaine intake. However, although pharmacological study on cocaine ivSA under FR1 can reveal involvement of neural substrates in reinforcement, we decided to focus on motivational properties of cocaine, since they represent an index of animals' wanting for the drug (Markou *et al.*, 1993). Indeed, PR schedule of reinforcement requires an exponential increase of the workload to get the reward (Hodos, 1961). Even though psychostimulant effects of cocaine can pharmacologically increase the value of the breaking point, PR schedules are far less sensitive to cocaine pharmacological effect than fixed-ratio schedules of reinforcement, and are currently used as models of some addictive-like behavior in rats (Deroche-Gamonet *et al.*, 2004). In our conditions, the breaking point under a PR schedule of intravenous self-administration was strongly reduced (about 40%), indicating that the orthosteric agonist LSP2-9166 compound was able to inhibit the reinforcing/motivational properties of cocaine. The LSP2-9166 did reduce cocaine reward-related effects probably through attenuation, via group III mGlu receptors activation, of the hyperglutamatergic state. Indeed, LSP2-9166 was demonstrated to be highly

potent at mGlu4 (EC_{50} = 0.06 µM) and mGlu7 (EC_{50} = 2.2 µM) (Acher *et al.*, 2012). This ligand also blocked morphine CPP expression and reinstatement after extinction (Hajasova *et al.*, 2018), an effect that was reversed by a selective mGlu7 antagonist, XAP044, suggesting a role of group III mGluR, and specifically mGlu7, in opiate rewarding effects. Interestingly, LSP2-9166 also reduced ethanol consumption, motivation for ethanol, and reinstatement (Lebourgeois *et al.*, 2018). Altogether these findings suggest that mGlu7 and mGlu4 activation represent a valuable strategy to block the effects of distinct drugs of abuse and in different behavioral rodent models, including operant paradigms. The effects appear drug-reward specific as natural-reward (sucrose consumption or sucrose self-administration) was not altered by LSP2-9166, indicating that the hedonic state of the animals was not affected by the mGlu compound (Hajasova *et al.*, 2018; Lebourgeois *et al.*, 2018). Noticeably, LSP2-9166 was more efficient in Hajasova et al.'s study since effects were already observed at lower doses (0.5 mg/kg, ip) than in our conditions (5 mg/kg, iv). In a pilot study (data not shown), we found that LSP2-9166 did not affect cocaine ivSA under PR schedule of reinforcement at successive doses of 0.1, 0.5, 1 and 2 mg/kg in rat. The reasons which could explain such discrepancies between studies are still unclear and may be related to differences in species (mice versus rats), behavioral paradigms (place preference versus intravenous self-administration), and/or the nature of the drug studied (morphine versus cocaine).

Our results are in accordance with previous publications showing that mGlu7 agonist AMN082 could reduce cocaine intake under progressive ratio schedule of reinforcement (Li *et al.*, 2009). Nevertheless, the cerebral mechanisms involved in this action are not clear. One hypothesis could be that LSP2-9166 effects on motivation of animals for cocaine, which can be visualized from the first day of treatment and maintained through repetitive treatment before each behavioral session, are mediated by a reduction of the glutamatergic neurotransmission in striatal regions, such as the NAc. By increasing the activity of mGlu4/7 located in presynaptic terminals of glutamatergic afferents coming from PFC, Amy and/or HPC, LSP2-9166 could lower glutamate release. Consequently, this would decrease the strength of goal-directed behavior of the animals to obtain the drug. Another possibility is that the effects of LSP2-9166 are mediated by a reduction of GABA release in NAc neurons targets, such as ventral pallidum and/or pars reticulate of the substantia nigra. Future electrophysiological or neurochemical studies will investigate these aspects on cocaine-induced glutamate neurotransmission and could be further completed with pharmacological studies using selective mGlu7 antagonists, which are still not available.

Our results on locomotor activity have to be taken into account in the interpretation of the effects of LSP2-9166 in rat motivation for cocaine. We observed a decrease of locomotor activity following a single injection of LSP2-9166 (15 min before exposing the rat to the device), which lasted for 40 min. Such a transient hypolocomotor effect was also observed at high dose in

mice (Hajasova *et al.*, 2018) or following icv injection in rats (Lebourgeois *et al.*, 2018). Noticeably, in the latter studies as well as in our conditions, rats were not exposed to the environment before the administration of the mGlu compound, which suggests that locomotor activity reflects both spontaneous locomotion and reaction to novelty. Interestingly, mGlu4 KO mice showed a higher basal locomotor activity (Blednov *et al.*, 2004), while activation of mGlu7 by AMN802 decreased the activity in rodents (Palucha *et al.*, 2007; Salling *et al.*, 2008). No effect on locomotor activity was reported following LSP1-2111, an mGlu4 preferential orthostetic agonist (Zaniewska *et al.*, 2014). These results may differ between mice and rats, but also depending on the dose and mode of administration of the mGlu ligand. Together with our findings, it appears that LSP2-9166 targeting mGlu4/7 had no major effect on locomotion that would drastically impact further behavior. LSP2-9166 did no impact sucrose self-administration with similar level of responding as for ethanol (Lebourgeois *et al.*, 2018), indicating that the behavioral response of the rats was not altered. In addition, no major motor side effect was observed in mice both on water and sucrose consumption (Hajasova *et al.*, 2018). Moreover, LSP2-9166 did not modify spatial memory in mice at the dose tested, supporting that any effect on locomotor activity did not strongly alter other behaviors (Hajasova *et al.*, 2018).

Interestingly, studies using mutant models for mGlu4 or mGlu7 receptors have highlighted a role for these receptors in several behavioral responses, including reward-related aspects. Reports have revealed that mGlu7 KO mice showed an increased alcohol consumption compared with littermates (Gyetvai *et al.*, 2011), findings that were confirmed by knock-down approaches using shRNA, showing an increase in alcohol intake and CPP (Bahi, 2013). This indicates a role for mGlu7 receptor in drug-associated behavior. Other studies using genetically modified animals highlighted a role in memory process, with mGlu7 KO mice showing an impaired working memory (water or radial arm maze) (Callaerts-Vegh *et al.*, 2006), and in contrast, mGlu4 KO mice showing better performance in the radial arm maze test (Iscru *et al.*, 2013). Other roles have been proposed with the findings of a stress-protective phenotype in mGlu7 KO mice (Peterlik *et al.*, 2017) or reduced anxiety-like behavior following shRNA induced knock down (O'Connor *et al.*, 2013). It would therefore be of interest to examine whether the LSP2-9166 compound could also impact on such behavioral responses.

It has been clearly established that relapse in humans is one of the most important problem in cocaine addiction (D'Sa *et al.*, 2011) and that glutamate neurotransmission is clearly involved in this aspect of the pathology (D'Souza, 2015). Therefore, an obvious perspective following our study is to evaluate the influence of LSP2-9166 in reinstatement of cocaine seeking after withdrawal and/or extinction. We hypothesize that activation of mGlu4/7 could increase extinction of cocaine-seeking behavior or reduce cue-induced cocaine-seeking within the recent concept of reconsolidation of cue memories. Indeed, mGlu7 activation with AMN082 has been shown to lower acquisition of cued fear conditioning and to heighten its extinction (Toth *et al.*,

2012; Fendt *et al.*, 2013). This effect targeting mGlu7 was even stronger than the administration of a NMDAR agonist, targeting NMDA receptor, known to be involved in this process (Toth *et al.*, 2012). Also, mGlu7 inhibition using knock down approach blocked fear extinction (Fendt *et al.*, 2013), and mice deficient for mGlu4 showed enhanced amygdala-dependent cued fear conditioning (Davis *et al.*, 2012, 2013). These data support a role for mGlu4/7 signaling in these learning and memory processes. As altering the state of cue-associated memories after their recall could reduce cocaine seeking (Gisquet-Verrier & Riccio, 2018; Monfils & Holmes, 2018), targeting mGlu4/7 with LSP2-9166 or related ligands represents therefore a promising strategy to reduce vulnerability to relapse.

References

The references are available at the end of the thesis manuscript.

Figure Captions

Figure 1.

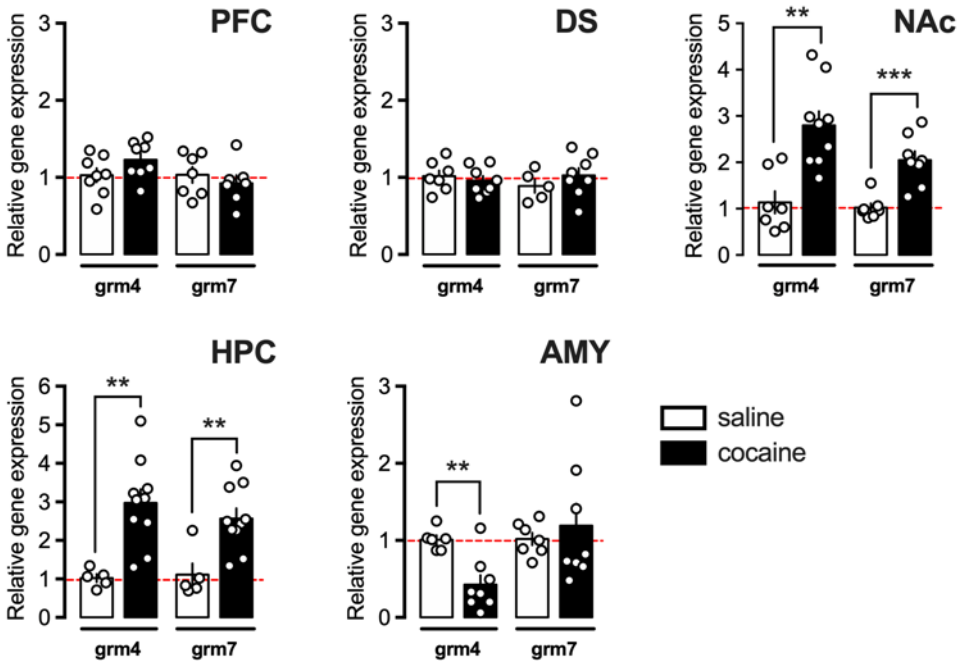

Figure 1. Cocaine effect on mGlu group III receptor expression. Level of transcripts for mGluR4 and mGluR7 were examined by qPCR in distinct brain areas following cocaine self-administration. Bars represent mean (+SEM) fold change vs saline group (n=5-10/group) for each receptor. *$P < 0.5$; **$P < 0.01$; ***$P < 0.001$.

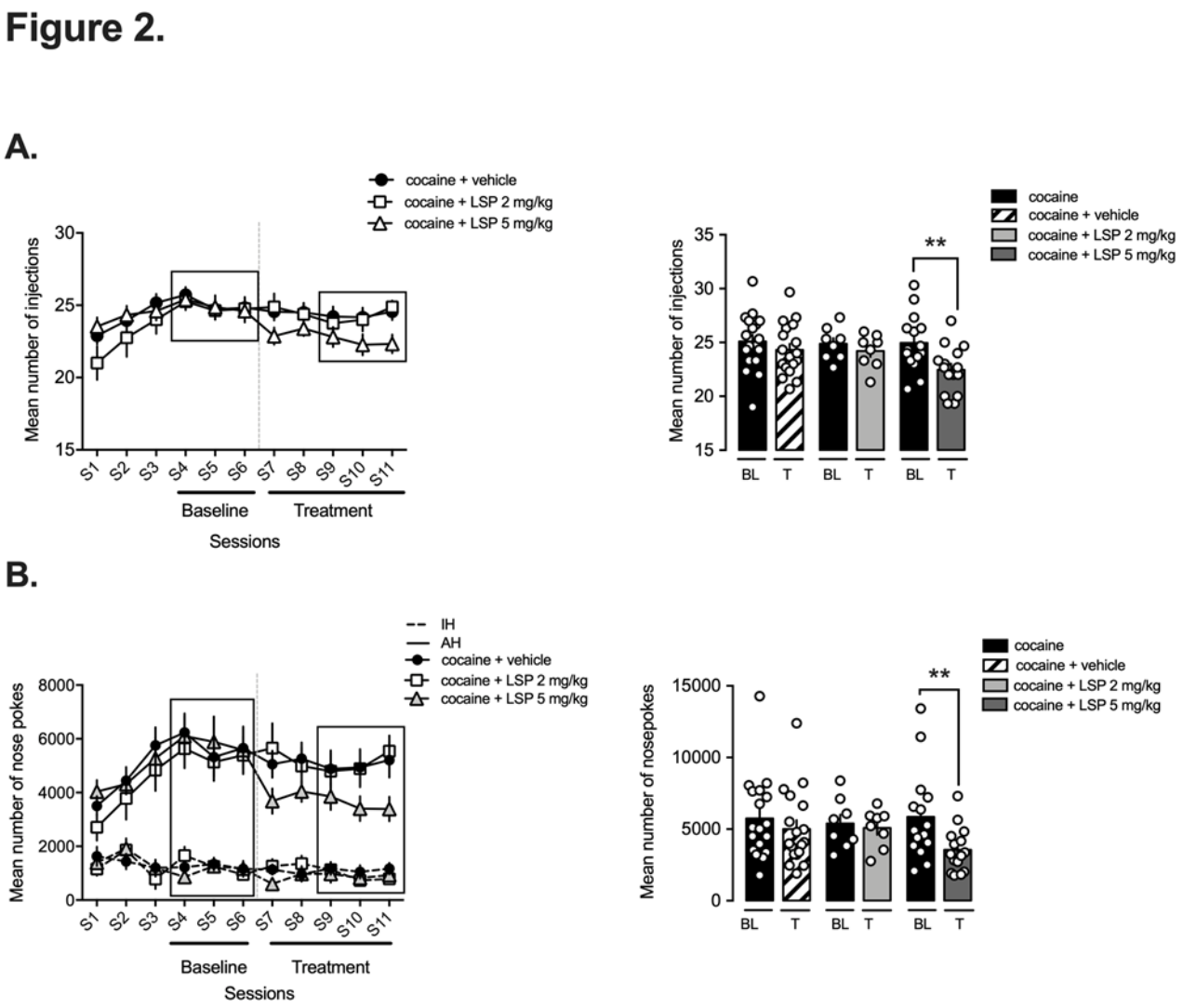

Figure 2. Effect of LSP2-9166 on cocaine-self administration. Effect of LSP2-9166 (2 and 5 mg/kg iv injections, 15 min before the operant session) on cocaine self-administration in a progressive ratio schedule of reinforcement. Mean (+SEM) number of injections (**A**) and nose-pokes (**B**) are presented across all sessions, with inactive NP indicated in dashed lines. Bars (black,

cocaine; hatched, cocaine + vehicle; light grey, cocaine + LSP2-9166 2 mg/kg; dark grey, cocaine + LSP2-9166 5 mg/kg) represent quantification of mean treatment effect (3 last sessions, S9, S10, S11) compared with mean baseline (3 last sessions S4, S5, S6) indicated by boxes in the left panels. ***P* < 0.01. (vehicle, n=18; LSP2-9166, 5mg/kg, n=15; LSP2-9166, 2mg/kg, n=8).

Figure 3.

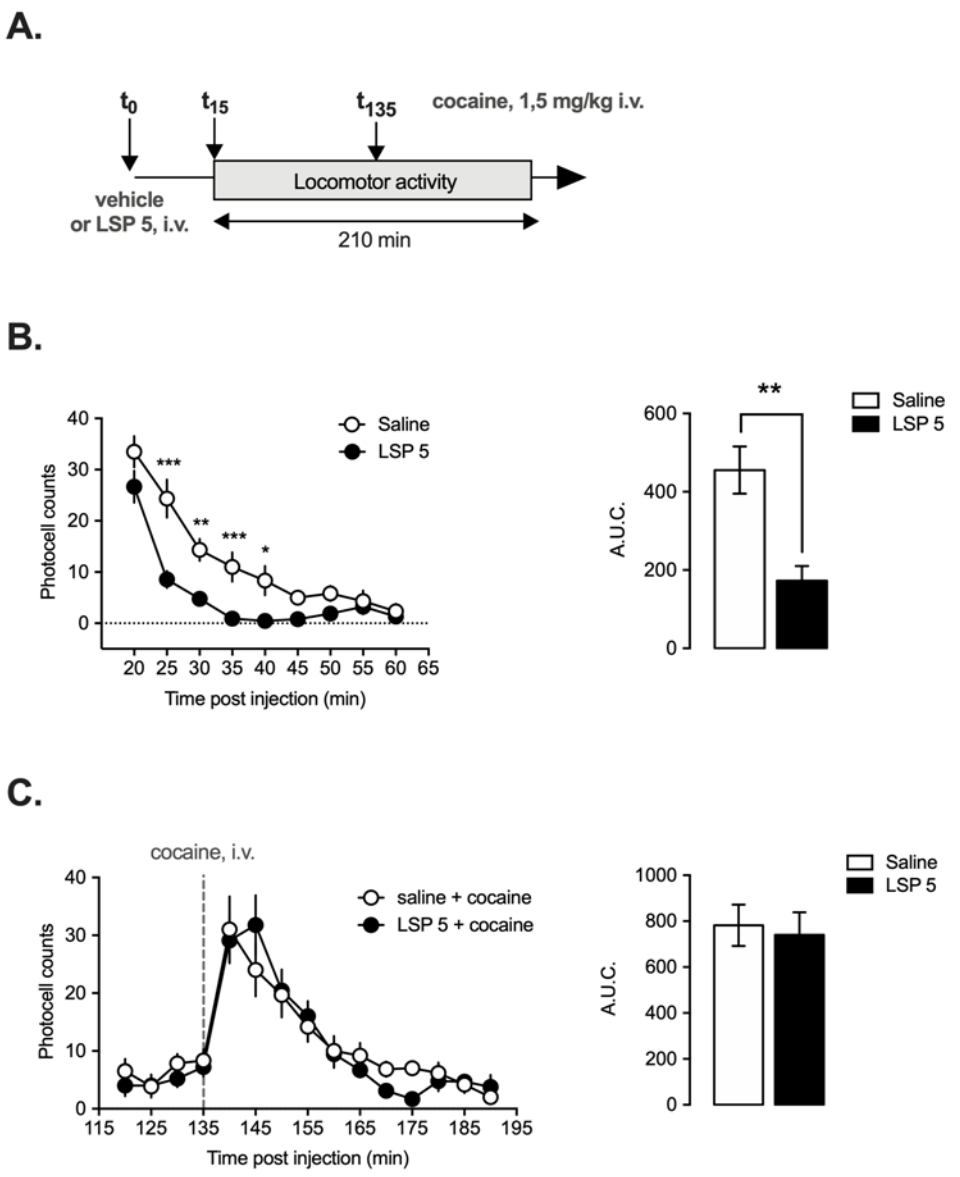

Figure 3. Modulation of locomotor activity by LSP2-9166. Experimental timeline for locomotor activity (**A**). LSP2-9166, 5mg/kg or vehicle was administered 15 min (t15) before

recording, and locomotor activity was measured for 120 min. Then, cocaine-induced hyperlocomotion was evaluated (t135) for 90 min. Locomotor activity was measured for 210 min in total. LSP2-9166 significantly decreased locomotor activity up to 40 min post injection (**B**). Cocaine induced similar hyperlocomotor activity in both control and LSP-9166 treated groups (**C**). **P* < 0.5; ***P* < 0.01; ****P* < 0.001. (vehicle, n=6; LSP2-9166, 5mg/kg, n=9).

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Author's contribution

PR, JZ, KB conceived the study and wrote the paper. PR, DN, CQ, DF performed the experiments. PR, DN, RB, KB analyzed the data and designed the figures. CC contributed to the discussion of the data and writing of the discussion. FA designed and synthesized the mGlu compound. All authors approved the final version of the manuscript.

Discussion

E. Discussion

I have discussed the different findings of my work in our distinct papers. Here, I would like to discuss specific questions in more details.

1. Is the hippocampus underrated within the context of cocaine intake?

Our most striking results are the marked modifications of the ECS occurring in the HPC following cocaine voluntary intake. these alterations were quite unexpected, maybe because the HPC is not considered one of the primary targets in cocaine addiction. For instance, a quick research on PubMed when I wrote this paragraph (September 2019) indicated 3057 publications regarding cocaine & NAc while only 775 are about cocaine & HPC **(Figure 23)**.

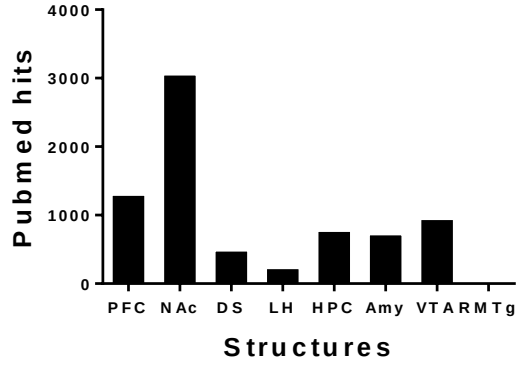


Figure 23: Number of studies regarding cocaine and brain structures. *Results from PubMed obtained with the following key words: "cocaine & prefrontal"; "cocaine & nucleus accumbens"; "cocaine & dorsal striatum"; "cocaine & lateral hypothalamus"; "cocaine & hippocampus"; "cocaine & amygdala"; "cocaine & ventral tegmental area"; "cocaine & rostromedial tegmental nucleus" on 09/30/2019.*

Noteworthy, HPC is essential to form associations between cues and drug context (Grant *et al.*, 1996; Childress *et al.*, 1999; Kilts *et al.*, 2001; Wexler *et al.*, 2001). Drug-associated memories persist overtime and are even resistant to extinction, as stimulation of the HPC or re-exposure to contextual cues-associated with drug reward reinstates extinguished drug self-administration in rats (Kutlu & Gould, 2016). Moreover, all drugs of abuse induce cognitive deficits in HPC-dependent learning and memory tasks, and they also decrease HPC plasticity, especially during withdrawal (Kutlu & Gould, 2016). This is the reason why, understanding the molecular and

functional changes occurring in the HPC in response to cocaine intake are crucial for deciphering these processes. Unfortunately, we did not have the time or the ability to investigate cognitive deficits nor plasticity changes occurring in the HPC following cocaine-SA (see *"**Article 1:** Voluntary cocaine intake modulates the endocannabinoid system in hippocampus"*). Nonetheless, we can speculate about expected modifications occurring in the HPC following voluntary cocaine intake. In our study, we argue that voluntary cocaine intake in rats enhanced the ECS which may be responsible for the electrophysiological changes occurring in HPC following cocaine intake. Indeed, chronic cocaine (either cocaine-SA or i.p) enhanced LTP in the HPC after a short withdrawal (Thompson *et al.*, 2004; Guan *et al.*, 2009). Interestingly, the ECS is described as a hippocampal network administrator as this system regulates HPC synchronized activity (Lupica *et al.*, 2017). One could easily perform cocaine-SA in rats, then records LTP (or LTD) in dorsal HPC when adding acutely specific CB1 or CB2 ligands at different stages of cocaine addiction (acquisition - extinction/withdrawal – reinstatement). As LTP is enhanced following cocaine-SA and CB1 activity is increased as our study showed, I would expect an even stronger enhancement of LTP following the activation of CB1, reversed by adding a CB1 antagonist. I am convinced that conducting this experiment would bring new insights providing a better understanding the role of the ECS in cocaine addiction. To go even further, I strongly suggest to also investigating the role of the endocannabinoid enzymes in the HPC. Indeed, we observe a strong increase of all ECS enzymes in HPC following cocaine-SA. Using inhibits of eCBs degradation (JZL184 or URB597), I would also expect an enhancement of LTP the in HPC, also reversed by a CB1 antagonist. Finally, we focused our study on the dorsal HPC which is involved in drug contextual associations (Fuchs *et al.*, 2005, 2007). On the other hand, ventral HPC is also involved in context and cue induced reinstatement of cocaine (Rogers & See, 2007; Lasseter *et al.*, 2010). Therefore, it would have been interesting to also investigate changes in the ventral HPC. This would allow to determine whether the ECS is involved in both contextual and cues associations or only in contextual associations.

2. Issues around the ECS

i. Other potential strategies to target the ECS ?

Giving the widespread expression of CB1 in the brain, clinical trials using a CB1 antagonist to treat obesity discovered side effects. These disappointing results make the use of CB1 ligands limited. Recently, clinical trials using CBD indicated positive results such as reduction of cannabis use and cannabis-induced withdrawal symptoms in individuals with cannabis dependence (Trigo, Lagzdins, *et al.*, 2016; Trigo, Soliman, *et al.*, 2016; Trigo *et al.*, 2018). As stated previously, preclinical studies regarding cocaine intake in rodents demonstrated that CBD attenuate cocaine-

SA in rodents (Xi *et al.*, 2011; Zhang *et al.*, 2014; Luján *et al.*, 2018; Galaj *et al.*, 2019). To date no clinical trials investigated the effects of CBD with other drugs of abuse but pre-clinical evidences are encouraging.

Targeting specifically eCBs enzymes for both biosynthesis and catabolism is challenging giving their involvement in other pathways (Di Marzo, 2018). For instance, a lipidomic profiling in Nape-Pld-KO mice indicated that several lipids levels were reduced in these mice (Leishman, Mackie, *et al.*, 2016). Only recently, inhibitors of 2-AG biosynthesis have been developed (Hsu *et al.*, 2010; Appiah *et al.*, 2014; Greig *et al.*, 2016) but no studies investigated the impact of inhibitors of eCBs biosynthesis on drugs of abuse to my knowledge. On the other hand, few studies investigated the effects of inhibitors of eCBs catabolism regarding drugs of abuse and results are still mixed for cocaine. Indeed, while URB597 attenuated cocaine-seeking in rats it did not decreased cocaine reinstatement in squirrel monkeys (Justinova *et al.*, 2008; Adamczyk *et al.*, 2009). More recently, mice with invalidation of fatty acid binding protein (potential AEA transports) exhibited an attenuated stress induced cocaine-CPP (Hamilton *et al.*, 2018). Altogether, research investigating the relationships between eCBs enzymes and drugs of abuse is largely undervalued. Nonetheless, this research deserves more attention giving the potential of these drugs. As our results indicated a strong enhancement of eCBs following cocaine-SA in the HPC, I would propose to evaluate the impact of DAGLα and/or NAPE-Pld inhibitors directly into the HPC of animals undergoing cocaine-SA to investigate whether this would modulate cocaine intake and seeking. However, these compounds are either inexistent or only recently developed as stated above. Nonetheless, as URB597 decreased cocaine-seeking in rats (Adamczyk *et al.*, 2009), I also propose to evaluate the impact of URB597 and/or JZL184 on cocaine intake and seeking when injected only into the HPC. In these conditions, I would expect an enhancement of cocaine intake and seeking. Indeed, an increase of eCBs levels enhance memory facilitation (*see **IV**: The endocannabinoid system; **10**: Involvement in pain, memory and learning; **ii**: Memory and learning*) , therefore I would expect a facilitation of intake and seeking. However, changing the context of the animals during cocaine-SA or seeking could reverse this effect.

Interestingly, several of these ECS enzymes inhibitors showed anti-obesity properties (Bisogno *et al.*, 2013; Baggelaar *et al.*, 2015). Moreover, Daglα-KO mice exhibited a hypophagic behavior even during a high fat diet which is very promising for future anti-obesity studies (Powell *et al.*, 2015). Regarding eCBs catabolism, many inhibitors of FAAH or MAGL have been already developed. Like for synthetizing enzymes, the lipidome is altered in both FAAH and MAGL-KO mice showing a broader involvement for catabolic enzymes (Leishman, Cornett, *et al.*, 2016). URB597 decreased food seeking in rats(Adamczyk *et al.*, 2009). Several clinical studies used FAAH inhibitors for their interesting potential on inflammatory pain (Di Marzo, 2018). However, a disastrous clinical study investigating the impact of a FAAH inhibitor on pain had to stop using one of these inhibitors upon five days of treatment as one of the four patients died from severe neurologic disorder, two

patients presented residual symptoms and the last one remained asymptomatic (Kerbrat *et al.*, 2016). This result was probably due to an unspecific effect of the inhibitor on FAAH (van Esbroeck *et al.*, 2017). Obviously, this slowed clinical research regarding FAAH inhibitors. Therefore, future clinical trials should assess whether these drugs are safe to use in obese patients.

ii. Opposite mRNA regulation for CB1 & CB2

In our study (***Article 4:*** *Activation of cannabinoid CB2 receptors induces expression of the epigenetic factors MeCP2 and HDAC2 in rat striatum*), we noticed a differentially gene expression of both cannabinoid receptors CB1 and CB2. Indeed, CB1 gene expression was increased by cocaine-SA in NAc, DS and HPC while CB2 mRNA decreased in PFC, DS and LH. There was a clear opposite regulation occurring in DS. This is not the first report of an opposite expression. For instance, CB1 gene expression increased in PFC and HPC while CB2 gene expression was decreased in both structures following cocaine injections during the adolescence of rats (García-Cabrerizo & García-Fuster, 2016). Moreover, CB1 and CB2 are differentially regulated during neuronal differentiation. Indeed, CB1 gene expression increased while CB2 decreased in neural progenitors. The role of CB1 has been largely investigated while the role of CB2 remains unclear in neuronal lineages (Galve-Roperh *et al.*, 2013). I propose that these results might be due to CB1 and CB2 expression on different cell types. Indeed, CB1 is mainly expressed on GABAergic and glutamatergic neurons while CB2 is expressed in microglial cells and dopaminergic neurons (CB2 expression in other cell types remain to be determined). In future studies, technologies enabling to cell sort specific cell types should provide a better understanding of such results and will be discuss later. Finally, as CB2 expression is up regulated in models neuroinflammatory models, CB2 gene expression decrease might indicate an involvement of neuroinflammatory processes that I discuss later.

iii. CB2 in the brain: is it functional?

In more than a decade of research regarding CB2 expression in the brain, many questions still surround its function in the brain. Stempel et al. indacted in their report a functional role for CB2 in the HPC. However, these results must be carefully viewed as the authors used an agonist of both CB1 and CB2 in one of the last experiment (see figure 7 in (Stempel *et al.*, 2016)). On the other hand, many studies focused on the role of CB2 in the VTA. Indeed, using mice with CB2 invalidation in dopaminergic neurons (CB2-DAT-KO), recent reports suggested that CB2 modulates motor activities, anxiety and the rewarding properties of alcohol and cocaine (Liu *et al.*, 2017; Canseco-Alba *et al.*, 2019). Recently, *ex-vivo* recordings in the VTA showed that JWH133, a CB2 agonist, induced a reduction in action potential firing (Ma *et al.*, 2019). Furthermore, administration of JWH133 directly into the NAc inhibited cocaine-enhanced extracellular DA and cocaine-SA, all blocked by AM630, a selective CB2 antagonist (Zhang *et al.*, 2017). Microinjections of JWH133 directly into the VTA also inhibited cocaine-SA in mice, which was reversed by AM630

(Zhang *et al.*, 2014). Taken together, these studies demonstrate that CB2 is indeed functional in the brain, at least in the VTA, and plays a role in reward responses.

In our cocaine study, we observed a reduction of CB2 gene expression in PFC, DS and LH while it remained unchanged in the binge-sucrose experiment. Altogether, our findings combined with the literature suggest that CB2 could participate to the maintenance of cocaine intake in rats. However, we must be very careful with such conclusions as the role and function of CB2 in PFC, DS or LH is still unknown. Giving the regulations we observed, an interesting approach would be to microinfuse JWH133 and/or AM630, during cocaine-SA and/or before cocaine seeking, directly in PFC, DS or LH to assess the potential impact of activation/blockade of CB2 in cocaine intake. Further studies are needed to ascertain the role of CB2 in reward-related brain regions.

iv. Are CB1 interacting proteins involved in our study?

As stated in *"IV: The endocannabinoid system"*, CB1 interacting proteins play a major functional role as CRIP1A and SGIP1 can both modulate CB1 signaling. Little is known about the relationship between SGIP1 and CB1 whereas the role of CRIP1a is better described. For instance, CRIP1A overexpression in the HPC increased CB1 activity (Guggenhuber *et al.*, 2016). Interestingly, in our study, we observed an enhancement of CB1 activity in the HPC along with an increase of CRIP1A and SGIP1 gene expression in HPC. Unfortunately, we were not able to measure CRIP1A and SGIP1 protein expression due to technical difficulties regarding CRIP1A specificity of the antibodies (see *"**C**: protocol optimization; **II**: Western blotting experiments: targeting cannabinoid receptors CB1 and CB2 and cannabinoid interacting protein"* and lack of antibody for SGIP1 staining. Thus, we cannot exclude their involvement in hippocampal CB1 enhanced activity. One possibility would be that CRIP1A protein expression is increased by cocaine which in turns, enhances CB1 activity. The role of SGIP1 needs to be studied further.

Similarly, MOP interacts with protein modulating its signaling such as calmodulin, periplakin and many regulators of G protein signaling (Georgoussi *et al.*, 2012). We did not investigate their expression in our study, but we cannot exclude their involvement in MOP enhanced activity that we observed in HPC.

v. A link between the endocannabinoid and opioid system: are both systems interacting with each other within the context of cocaine intake?

The ECS and OS share commonalities such as:

- Receptors coupled to inhibitory G protein
- Distribution throughout the brain overlaps
- Establishment of CB1-MOP & CB1-Delta heteromers in vivo

- Numerous studies report the synergistic interaction of the opioid and cannabinoid agonists (Nielsen *et al.*, 2017)

Interestingly, pharmacological blockade of either opioid or cannabinoid receptors attenuates behavioral responses induced by an agonist of the other system (Fattore *et al.*, 2005). For instance, Rimonabant decreases morphine-SA in mice while Naloxone attenuates cannabinoid-SA (Navarro *et al.*, 2001). Noteworthy, we observed an enhancement of both mRNA expression and functional activity of MOP and CB1 receptor in the HPC following cocaine-SA. These changes could be associated with the formation of MOP-CB1 receptor heteromer, as previously established in rat striatal membranes in postsynaptic neurons (Rodriguez *et al.*, 2001; Rios *et al.*, 2006). Furthermore, our data showing a marked increase of delta receptor in HPC could also argue for potential Delta-CB1 heteromers. This would have to be examined in details. Thus, as Delta-CB1 heteromers have been already found in cortical areas (Bushlin *et al.*, 2012; Rozenfeld *et al.*, 2012), these results need further attention as they may indicate a cocaine-induced increase of these heteromers in the HPC.

Targeting CB1-Delta or CB1-MOP heteromers as a therapeutic strategy for addiction is interesting. For instance, MOP and Delta also forms heteromers which can be targeted with specific ligands eliciting acute thermal analgesia comparable to morphine but induced less tolerance and physical dependence upon repeated administration (Daniels *et al.*, 2005; Gomes *et al.*, 2013; Pierre *et al.*, 2019). Regarding cocaine, its impact on D2-Sigma1 heteromers has been investigated however, there is no specific agonist of this heteromer to study its potential effect on cocaine intake (Beggiato *et al.*, 2017; Borroto-Escuela *et al.*, 2019). Thus, further studies are needed to assess the role of functional heteromers in the field of cocaine addiction as targeting specific heteromers appears as potential novel therapeutic targets.

3. Epigenetic prospects

i. Cocaine & histone modifications: which modification to study and where to look?

In *"**Article 1**: Voluntary cocaine intake modulates the endocannabinoid system in hippocampus"*, we demonstrated that histone modifications occurred at ECS gene promoters and exon 1 in HPC. Indeed, we have shown for the first time that cocaine triggers H3K27Ac alterations

and that ECS genes are regulated by H3K4Me3 and H3K27Ac histone marks. The relationships between psychostimulants and histones has already been reviewed (Nestler, 2014; Kalda & Zharkovsky, 2015).Here, I discuss several aspects in detail.

We focused on histone marks as cocaine is known to trigger such alterations (Nestler, 2014). Many studies investigated whether cocaine modulated histone acetylation or methylation, and mainly focused on NAc (Nestler, 2014). Interestingly, studies reported an increase of H3 and H4 acetylation in NAc of rats acutely or chronically injected with cocaine or amphetamine (Kumar *et al.*, 2005; Kalda *et al.*, 2007; Schroeder *et al.*, 2008; Shen *et al.*, 2008). More precisely, chronic psychostimulant exposure hyperacetylated H4 at c-Fos and FosB promoters in NAc (Kumar *et al.*, 2005; Renthal *et al.*, 2008). Histone methylation was also affected as several reports demonstrated an effect of chronic cocaine on H3K9Me2 and H3K9Me3 in NAc (Maze *et al.*, 2010, 2011). Overall, these findings describe that cocaine induce specific histone modifications leading to long-lasting gene expression changes. Therefore, it appears essential to decode these cocaine-induced alterations on the epigenome to better understand the histone signature following cocaine intake (Walker *et al.*, 2015).

HDAC inhibitors also strongly modulated cocaine rewarding properties. Indeed, HDAC inhibitors usually enhance the rewarding properties of cocaine (Kumar *et al.*, 2005; Schroeder *et al.*, 2008; Sun *et al.*, 2008; Wang *et al.*, 2010; Malvaez *et al.*, 2013). However, there are still some discrepancies regarding these results as other reports indicated an increase of motivation towards cocaine intake (Romieu *et al.*, 2008; Hitchcock *et al.*, 2019). Briefly, these discrepancies are probably due to many factors such as, the paradigm used (cocaine-CPP versus cocaine-SA), systemic injections versus micro infusions of the inhibitor. Regarding histone methylation, inhibition of one of the histone methylation enzyme attenuated cocaine CPP (Li *et al.*, 2015). Altogether, these studies provide further potential for treatment by epigenetic modulation.

As highlighted here, most of studies investigated cocaine-induced histone modifications in the NAc and only in mice (De Sa Nogueira *et al.*, 2019). Very interestingly, our study demonstrated similar adaptations in the HPC. These findings highlight that these mechanisms are potentially occurring in many other regions. Giving the involvement of the HPC in cocaine-SA as previously discussed, I strongly suggest that future studies should avoid focusing one only one structure such as the NAc and investigate other reward-related brain regions.

Moreover, our study also demonstrated that histone modifications can occur in several part of the gene body (promoter and exon). We focused our interest on CB1, DAGLα and FAAH; It would have been interesting to be able to screen across all ECS genes. To do so, the golden standard technic is to perform ChIp-Seq, which enable to investigate all genes potentially regulated, using one histone mark or a chromatin-binding protein. Obviously, we should have performed such approach in our conditions, however, the price of such technic is still quite high. As it is still

difficult to conclude on a cocaine signature on histone modifications (De Sa Nogueira *et al.*, 2019), it appears that only ChIp-Seq studies will explain the mechanisms involved in cocaine adaptations. Only then, we may have a clue regarding which modification to focus on. To date, we are still far from understanding cocaine-induced modification of the epigenome. Therefore, I strongly believe that only genome-wide approaches will effectively shed light on the epigenome complexity.

ii. Chromosome conformation capture: is this new line of research promising?

In our study aiming at better understanding cocaine-induced modifications on the ECS, we showed that CB1 promoter region interacted with several other chromatin regions in both HPC and NAc. Interestingly, cocaine modulated these interactions, particularly in the NAc *"**Article 1**: Voluntary cocaine intake modulates the endocannabinoid system in hippocampus"*. Development of chromosome conformation capture approaches (Davies *et al.*, 2017) applied to neuroscience has open new perspectives of research in addiction. These new technologies allow investigating physical interactions at the chromatin level, thus revealing potential regulatory elements at specific promotor sites. Further characterizing these regulatory elements (e.g. super-enhancers (Achour *et al.*, 2015; Le Gras *et al.*, 2017) using 3D genome investigation techniques will bring insight into mechanisms underlying neuroplastic adaptations in cocaine addiction (Engmann *et al.*, 2017). Such approach applied to the ECS or OS components could pave the way for therapeutic intervention targeting epigenetic modifications. For instance, CRISPR-Cas9 tools can be designed to act as epigenome regulators to either incorporate or recruit DNA or histone-modifying enzymes which add or remove a methyl group from proximal CpG sequences (Patsali *et al.*, 2019). For instance, a study designed a CRIPR-Cas9-based acetyltransferase to selectively acetylate H3K27 to boost specific gene transcription, even gene from proximal and distal enhancers (Hilton *et al.*, 2015). Thus, CRIPR-Cas9, zinc-finger protein and transcription activator-like effector nucleases are emerging as a high promising tool in the field of neuroscience to treat neurological disorder such as Parkinson (Kantor *et al.*, 2018), Alzheimer (Bustos *et al.*, 2017) or Huntington disease (Tabrizi *et al.*, 2019).

iii. Can epigenetic help find new treatment strategies? A transgenerational focus

During the winter of 1944, in the Netherlands, a large hunger affected 4.5 million people and 22 000 died. More than 6 decades later, studies demonstrated that the progeny of these people was significantly more affected by schizophrenia, depression but also diabetes, cardiovascular diseases, obesity or other metabolic disorders (Roseboom *et al.*, 2011). What could

be the mechanism(s) leading to these observations in subsequent generations? Several studies provided later the first direct evidences for epigenetic programming through prenatal famine exposure. These breakthroughs suggest that early undernutrition can cause epigenetic changes that persist throughout life (Roseboom *et al.*, 2011). For instance, offspring of mothers exposed to the Dutch Winter Hunger exhibited DNA methylation level alterations of imprinted genes such as the insulin-like growth factor II gene or genes involved in growth and metabolic disease such as leptin compared with their unexposed same-sex sibling (Heijmans *et al.*, 2008; Tobi *et al.*, 2009, 2014). In a similar context, children from Holocaust survivors may present epigenetic alterations but these evidences need to be further confirmed (Yehuda *et al.*, 2016).

Thus, exposure to a high and prolonged stress induced neuroepigenetic modifications which can be transmitted to the offspring, even in animal models. For instance, Isabelle Mansuy lab demonstrated that behavioral alterations induced by a paternal trauma exposure were transmitted until the fourth generation in mice (van Steenwyk et al., 2018). These behavioral changes were associated with an increase of glucocorticoid receptor expression in HPC associated with a decrease of DNAm in the promoter in mice (Gapp et al., 2016).

In our review (De Sa Nogueira *et al.*, 2019), we described reports (including candidate gene and genome-wide studies) which examined drug-induced epigenetic modifications transmitted to the offspring in animal models. Our analysis indicated that most of studies investigated the potential modifications in the F1 generation and unfortunately not further. Interestingly some human studies have explored such modifications from blood samples, but results may not reflect the neuroepigenetic alterations. However, very few studies investigated whether cocaine intake induced epigenetic modifications transmitted to subsequent generations. On the contrary, many studies examined the effect of obesogenic diet and exercise in offspring (Barrès & Zierath, 2016) but, to my knowledge, none examined whether sucrose intake could induce such changes.

Altogether, transgenerational studies are fascinating giving their importance for both research and society as they hold great potential in medicine, both to uncover disease biomarkers and therapeutic interventions (Nagy & Turecki, 2015). However, this field is still emerging and needs to be developed further.

4. The concerns about sugar

i. Commonalities and differences observed between cocaine and sucrose in our conditions

I presented earlier in section *"**III**: Binge-eating disorder: is it food addiction?; **4**: Common neurobiological basis of drug and food addiction"* the fact that some commonalities do exist between drugs of abuse and palatable food impact on the brain and behavioral outcomes. For instance, Nicole Avena established commonalities between the binge sucrose paradigm and addictive behaviors. In our study we directly compared both paradigms for ECS gene expression. We noticed only one common regulation between cocaine-SA and binge sucrose with the up-regulation of CB1 mRNA in the NAc. These results are discussed separately in each paper, but overall, our main finding is that binge-sucrose induced considerably fewer regulations on the ECS contrary to cocaine-SA in reward-related brain areas. Also, regarding eCB levels, we observed no commonalities in their regulation in the area investigated. In the RNA-Seq analysis conducted in the NAc of binge-sucrose animals, we have identified regulated genes previously described as involved in cocaine addiction such as MC4r and GRIA1. These transcript regulations represent therefore other examples of commonalities between binge-sucrose and voluntary cocaine intake.

Overall, despite few interesting commonalities occurring in NAc, we observe more differences between our paradigms of voluntary cocaine or sucrose intake. In the HPC for instance:

- CB1 gene expression remained unchanged by sucrose while cocaine-SA increased strongly CB1 mRNA
- 2-AG level is decreased in binge-sucrose animals while cocaine-SA enhance 2-AG levels
- We observe marked regulation of enzymes gene expression following cocaine-SA while their mRNA expression remains unaffected following the binge-sucrose paradigm

These differences occurred probably because, within the binge-sucrose paradigm, the HPC is involved in a far lesser manner. Indeed, as rats stayed in their home cage, the context remains the same. Furthermore, whether electrophysiological changes are occurring in the HPC following binge-sucrose intake is unknown. If so, the ECS might be not involve giving are result. At this stage of our project, it is difficult to speculate on the meaning of these differences. Thus, we need to further investigate the neuroadaptations occurring following binge-sucrose.

ii. Are sugar & food addictions real ?

The terms palatable food and sugar addiction are more and more often found in media or other networks. The work of Serge Ahmed demonstrated that sweetness has the potential to overcome reward induced by drugs of abuse. Indeed, in rats trained to self-administer cocaine (Lenoir *et al.*, 2007), nicotine (Huynh *et al.*, 2017) or heroin (Lenoir *et al.*, 2013) intravenously, all animals preferred to self-administer saccharin or sucrose (liquid sweeteners) when they had the choice.

Interestingly, in the intravenous cocaine study (Lenoir *et al.*, 2007), rats with choice between both cocaine and sweeteners (sucrose and saccharin) developed a preference for sweeteners far more rapidly compare to animals self-administering only sweeteners (day 2 versus day 7). As cocaine activates brain stress pathways, this raised the question whether the animals with choice between cocaine and sweeteners developed an aversive reaction towards cocaine (Ettenberg & Geist, 1991; Koob, 1999). Thus, giving the cocaine-induced stress possible reaction in these animals, one can expect them to trigger more responses toward sweeteners. To determine whether drug history influences the choice between saccharin and cocaine, the same authors (Serge Ahmed 's lab) offered the choice between sweeteners or cocaine to rats trained to self-administer cocaine. Very interestingly, the majority of rats quickly turned their responses towards sweeteners (Lenoir *et al.*, 2007), invalidating the theory that cocaine-induced stress would be involved in this behavior.

Serge Ahmed and his team observed this same behavior with nicotine-SA (Huynh *et al.*, 2017) and heroin-SA (Lenoir *et al.*, 2013). However, rats trained to self-administer heroin with extended access did not shifted towards sweeteners (Lenoir *et al.*, 2013), which highlight unique properties of drugs of abuse suggesting here that heroin is more addictive than other drugs. Another group showed also a preference towards sucrose pellets over methamphetamine-SA with almost a total absence of responses towards methamphetamine (Caprioli *et al.*, 2015). Another team using brain stimulation reward showed that rats also preferred sugar pellets compare to self-stimulation (McMurray *et al.*, 2017). Furthermore, the preference of saccharin and sucrose over drugs of abuse was unlikely associated to dopamine release as cocaine, for instance, is far more potent than sweeteners in inducing dopamine levels in NAc **(Figure 24)**. Thus, this suggest that other neurobiological mechanisms facilitate preference for sweeteners over drugs of abuse. An interesting target would be the opioid system, a well-known endogenous system which forms hedonic hot spots in the brain and modulates hedonic reward (Peciña *et al.*, 2006). For instance, our team recently showed that invalidation of MOP in mice decreased binge eating of a sweet solution (Awad *et al.*, 2019). It would be of great interest to study further whether the mechanisms involved in the override of natural reward versus drugs of abuse still occurs in models with opioid system impairments.

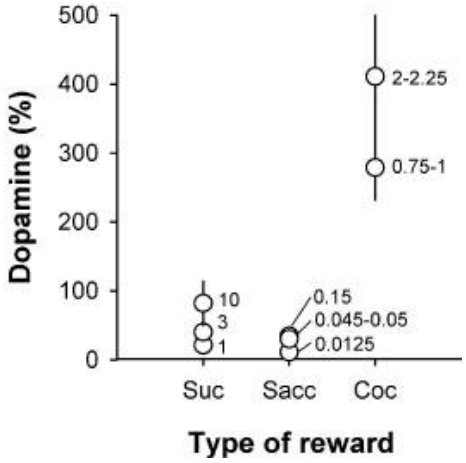


Figure 24: effects of sucrose, saccharin or cocaine consumption on NAc dopamine levels. *Mean (±S.E.M) levels of extra-cellular dopamine in the NAc (expressed as percent change from baseline) during sucrose, saccharin or cocaine intake. These results are based on a meta-analysis of the literature* (Lenoir *et al.*, 2007)*. Values that appear on the right of symbols represent sucrose or saccharin concentrations (in %) and cocaine doses (in mg/kg).*

Overall, these results raised several questions. First, these findings are groundbreaking as they demonstrate that sweetness surpasses the reward effect of drugs of abuse. Therefore, is sugar the new golden substitution therapy to treat individuals with addiction? Most likely no as a report indicate that rats exposed to sugar, continuously or intermittently during withdrawal following cocaine-SA, do not exhibit a reduction of cocaine seeking (Nicolas *et al.*, 2016). But on the other hand, sugar maybe is as addictive or even more addictive than drugs of abuse. Thus, should we avoid it and raise concerns regarding its dangers? Or finally, are these findings only applicable to rats or mice? Very interestingly, another putative addictive behavior is exercise. Marsicano group recently demonstrated that the motivation towards running overcome motivation to eat palatable food in mice, an effect dependent of CB1 (Muguruza *et al.*, 2019). Thus, as long as the mechanisms for motivation towards feeding are not elucidated, the situation will remain complex.

iii. The limits of cocaine-SA

Despite cocaine-SA being the golden standard to measure addiction-like behavior in rodents, animals often have only access to one reward without any access to another. In that context, even individuals without any vulnerability factors would be expected to self-administer

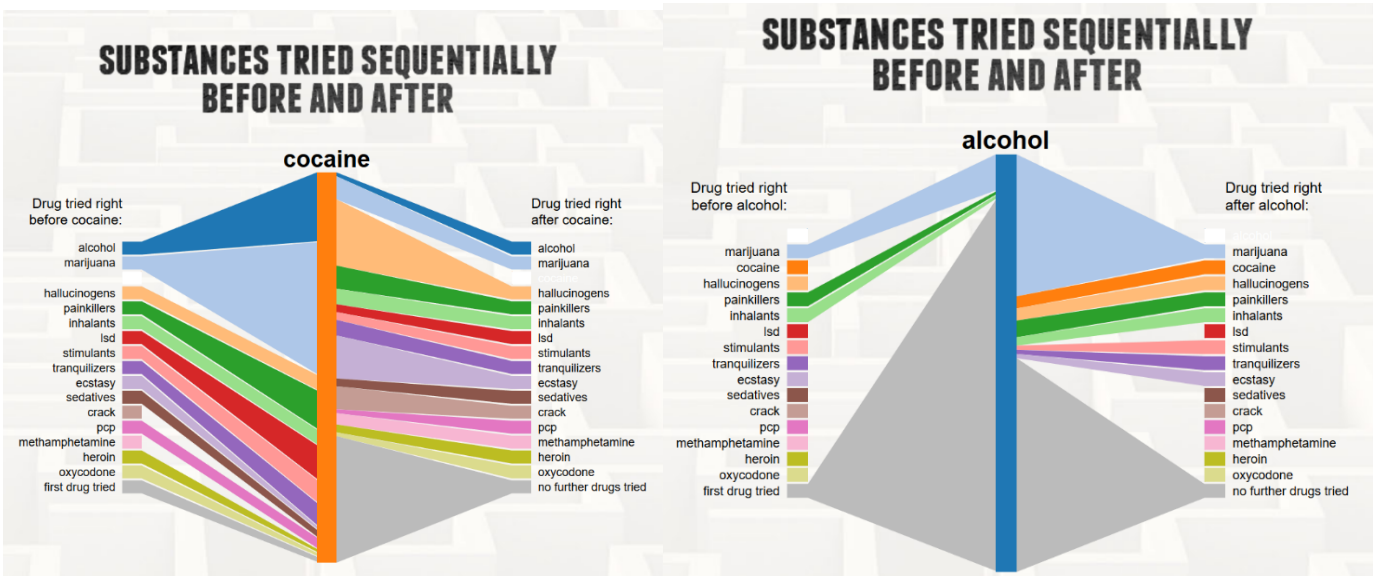
a reward if it was the only activity available. The famous "Rat Park experiment" illustrated that matter as rats in the Park hardly used the drug whereas single-housed animals used it compulsively until overdose (Alexander & Hadaway, 1982). Several studies have shown that enriched environment prevented vulnerability towards cocaine intake/addiction (Solinas *et al.*, 2010; Nader *et al.*, 2012). The best cited example in humans is the Vietnam War. Indeed, 35% of American soldiers actually tried heroin while in Vietnam, and 19% became addicted to it (Robins *et al.*, 2010). Interestingly, of all the soldiers addicted back from Vietnam, only 12% relapsed over a 3 years period after their return (Robins *et al.*, 2010) indicating the powerful effect of a new and enriched environment. Thus, the environment in cocaine-SA experiment must not be underestimated and, ideally, cocaine-SA studies should include an enriched environment in their paradigm to closer to human conditions. However, modeling the effect of an enriched environment with more animals in the same environment and through an intravenous route is obviously highly challenging and would necessitate novel and advanced technologies. Indeed, the catheter surgery makes impossible to perform this experiment yet. This is the reason why other studies added a non-drug alternative such as sucrose or saccharin in their set up experiment (Ahmed, 2018) instead of changing the environment. These studies are discussed below.

iv. Is sugar a gateway drug?

The term "gateway drug" has been proposed by Robert Dupont, a psychiatrist and anti-drug activist, in the 80's (Kandel, 2002). The gateway drug theory proposes that psychoactive drugs are associated with an increased probability of using further drugs. In other words, some drugs serve as gateways for the use of other drugs (Kandel, 2002). For instance, a recent study conducted in the US indicated that individuals who started with cannabis before using any other substance had a lifetime cumulative probability of 44.7% to progress to other illicit drug use (Secades-Villa *et al.*, 2015). With the increase of vaping worldwide, studies are now proposing that e-cigarette could serve as a gateway tool to progress to cigarettes or cannabis. However, this hypothesis is still premature giving e-cigarettes have not been available for a long enough time period (Fadus *et al.*, 2019). A final example would be that 72% of people who have ever used cocaine tried cannabis first, whereas only 1% of cannabis users tried cocaine first **(Figure 25)**. This highlights the idea that certain drugs lead to future use of another. However, correlation is not causation.

A survey analysis ran by the American Addiction Centers asked over one thousand Americans what was their first ever substance used until their tenth substance used. Alcohol, tobacco, and marijuana are most often the first three substances tried **(Figure 26)** (https://americanaddictioncenters.org/the-real-gateway-drug**)**. Over 60 percent of people said alcohol was their first substance ever used. Drugs like cocaine, crack, methamphetamine and hallucinogens are often used as the fourth to sixth substance used. This is in contradiction with

the common belief that cannabis is “The” gateway drug even if cannabis appears as the second one. Thus, it seems that alcohol may be the “real” gateway drug, probably because of its higher availability. Indeed, availability of drugs may help explaining the higher risk of progression to illicit drugs of abuse. A study found that 40% of lifetime cannabis users progressed to other illicit drug use, highlighting the potential dangers of policies that may increase the availability of cannabis (Secades-Villa *et al.*, 2015).



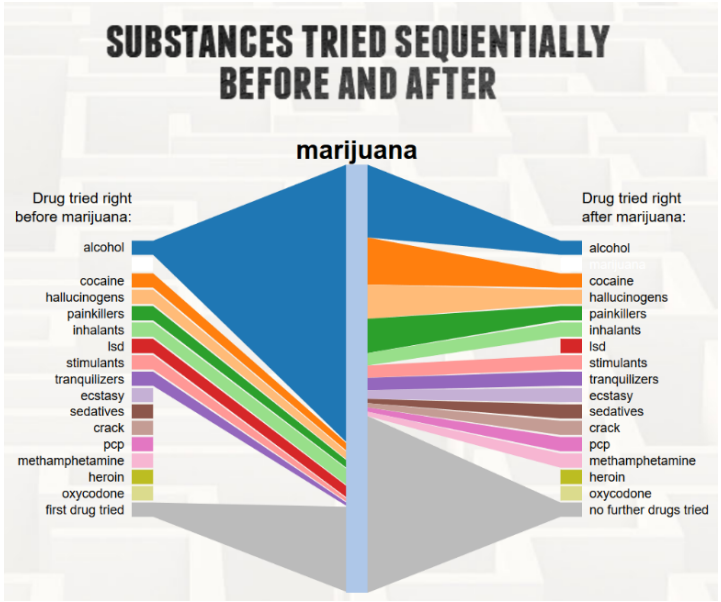


Figure 25: representation of substances used before and after Cocaine, Alcohol and Marijuana. *Data available on the website https://www.treatment4addiction.com which offers an interactive way to look at the use of drugs before or after most common substances used. Data are from a survey analysis from the National Survey on Drug Use and Health conducted in 2012.*

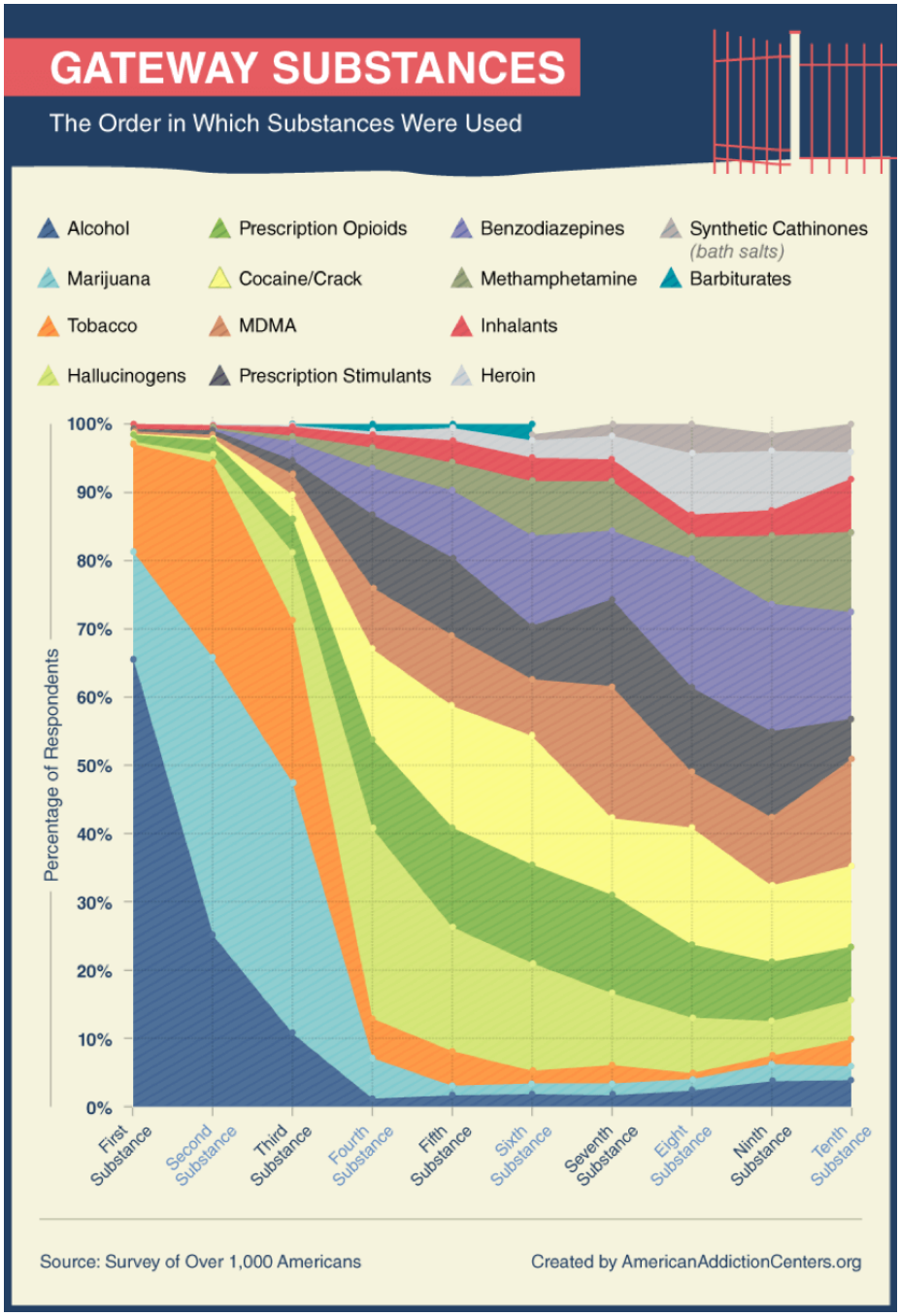


Figure 26: Order in which substances were used. *Survey of 1,057 Americans asked to answer questions related to their substance use history. Thirty-eight percent of respondents identified as women, 61 percent of respondents identified as men, and 1 percent identified as a gender not listed by the survey.*

The group of Nicole Avena investigated whether a history of high sugar intake promotes future drug intake, therefore evaluating the gateway hypothesis for sugar intake in rats. Interestingly, rats which previously binged on sucrose exhibited a higher intake of ethanol compared with other

groups. Conversely, rats with a previous history of ethanol consumed more sugar (Avena *et al.*, 2004). In the same way, rats exhibited an increase of fentanyl and amphetamine preference following 3 weeks of access to sugar (Vitale *et al.*, 2003). Still with opiates, an intermittent access to sucrose (2h in the morning, 2h in the afternoon) before morphine-CPP induced attenuated CPP but no differences in terms of extinction and reinstatement (Zhai *et al.*, 2008). However, more recently, rats which binged on sugar or had continuous access still displayed morphine-CPP (Smail-Crevier *et al.*, 2018). Interestingly, rats on the bingeing paradigm did not display sucrose-CPP suggesting that sucrose decreases reward perception (Smail-Crevier *et al.*, 2018).

Previous studies have shown that sucrose bingeing enhances the locomotor responses to cocaine and amphetamine (Avena & Hoebel, 2003; Gosnell, 2005). In female rats with access to sugar in a non-bingeing manner, they display the same pattern of cocaine sensitization compare to controls suggesting that only a binge paradigm would enhance cocaine sensitization (Serafine *et al.*, 2015). Furthermore, a history of intermittent access to glucose impaired cocaine-CPP in rats whereas the same bingeing paradigm with sucrose and fructose did not prevent cocaine-CPP indicating that sweeteners may affect differently the perception of reward (Rorabaugh *et al.*, 2015). In a study where rats had access to sucrose pellets 1 hour per day for a week before self-administering cocaine, the author identified a high-sucrose intake group among the sucrose eaters. Interestingly, this group, after a mild stressor (saline injection) slightly consumed more cocaine, but this effect was not consistent through the whole experiment. This observation was not reproduced with fat (Gosnell, 2000). These findings suggest that sugar intake, in combination with a stress factor, promotes drugs of abuse intake.

Altogether, these findings above suggest that binge-sucrose modulate cocaine intake in rats and vice versa but are dampened by another study indicating that when rats are exposed to sugar only during their adolescence, they display the same intake of cocaine compare to controls in adulthood, suggesting no gateway effect (Vendruscolo *et al.*, 2010).

In conclusion, the most studies strongly suggest an effect of sugar on reward perception. Further studies are needed, especially regarding the impact of a sucrose bingeing pattern on voluntary cocaine intake. For instance, conducting a binge-sucrose paradigm right before cocaine-SA (and vice-versa) should bring new insights regarding that question. Giving the literature, I would except that binge-sucrose prior cocaine-SA should enhance cocaine-SA in rats.

5. Are inflammatory processes involved in cocaine or sugar addiction?

The relationships between addictive disorders and neuroinflammation remain unclear. However, several reports indicate a potential role for neuroinflammation in that context (Clark *et al.*, 2013; Ersche & Döffinger, 2017; Lacagnina *et al.*, 2017; Kohno *et al.*, 2019). For instance, non-selective reduction of neuroinflammation with the phosphodiesterase inhibitor Ibudilast reduced methamphetamine, opioid, and cocaine seeking (Beardsley *et al.*, 2010; Schwarz & Bilbo, 2013; Snider *et al.*, 2013; Charntikov *et al.*, 2015; Poland *et al.*, 2016) suggesting a strong link between neuroinflammation and drugs of abuse.

In cultured A172 astrocytoma cells and primary human astrocytes, cocaine induced the activation of astrocytes associated to a sequential activation of endoplasmic reticulum stress and increased the expression of proinflammatory mediators such as TNF, IL1B, and IL6 (Periyasamy *et al.*, 2016). These findings suggest that cocaine induce neuroinflammatory processes in the CNS. More recently, in rats trained to cocaine-SA, toll-like receptor 4 (known for inducing proinflammatory signaling in the CNS) pharmacological blockade in the VTA reduced cocaine-primed reinstatement but had no effect on sucrose seeking (Brown *et al.*, 2018). Interestingly, cocaine-SA increased mRNA expression IL1B in the VTA. Even an acute cocaine injection increase IL1B in NAc (Cearley *et al.*, 2011). Moreover, a recent study from our team suggest that neuroinflammatory pathways involving NFκB are activated in PFC following cocaine-SA in rats (Fonteneau *et al.*, 2017). Altogether, these findings above strongly suggest that cocaine mediates the induction of neuroinflammation in the CNS. Therefore, we cannot exclude a link between addictive behaviors and neuroinflammation in our study. Interestingly, CB2 is crucially involved in neuroinflammatory pathways as its expression strongly increase in neuroinflammatory models (Cassano *et al.*, 2017). CB2 gene expression decreased in our own study suggesting a decrease of neuroinflammatory processes which is contradiction with studies above. However, speculating on the role of CB2 here is difficult as we did not investigate CB2 protein expression.

Drugs of abuse seems to be involved with neuroinflammatory processes but what about palatable food? Interestingly, a 3 months high fat-sucrose diet also increased many proinflammatory factors as well as NFκB transcriptional activity in NAc (Décarie-Spain *et al.*, 2018). However, 30 days of sucrose intake did not impact neuroinflammatory markers (Hsu *et al.*, 2015). I did not find any other clear signs of sucrose-mediated neuroinflammation in the literature. However, obese individuals exhibit a neuroinflammation which leads to cognitive impairments (Miller & Spencer, 2014). Thus, we cannot exclude the fact that sucrose may induce neuroinflammation, at least in some brain structures. An interesting approach would be to screen for cytokine (both pro or anti-inflammatory) expression using a cytokine array ELISA kit, in reward-related brain regions after both cocaine-SA and binge-sucrose to identify potential neuroinflammatory process.

6. Future directions

i. Sex differences

We performed all our studies on male rats. It would have been very interesting to evaluate all the molecular changes in both sexes for several reasons. Indeed, as previously introduced in section *"**III**: Binge-eating disorder: is it food addiction?, **1**: General aspects"*, among individuals with eating disorders, there is majority of females. Also, females with addictive disorders in general are more prone to relapse. More importantly, alterations of the ECS appears to be sex specific. For instance, women exhibit higher CB1 expression than males in human blood cells (Onaivi *et al.*, 1999). Moreover, women and males respond differently to cannabinoids. For example, males are more sensitive to the hyperphagic and hypophagic effects of the CB1 activation (Diaz *et al.*, 2009) (for review see (Fattore & Fratta, 2010; Craft *et al.*, 2013)). Moreover, females are more sensitive to cannabinoid-induced antinociception and males and females differs in terms of cannabinoid regulation of energy homeostasis (Wagner, 2016). A recent study demonstrated that eCBs increased microglia-induced phagocytose in the Amy of male rats compare to females. Very interestingly, this sex specific difference promoted juvenile social play only in males (VanRyzin *et al.*, 2019). Finally, a study found that adolescent exposure to cocaine affected differently CB1 functionality and CB2 expression in several reward-related brain regions in rats according to the sex (Llorente-Berzal *et al.*, 2013) in structures such as the PFC, NAc, HPC and VTA. Thus, these findings highlight the importance of studying both sexes in addictive and eating disorders, especially when the ECS is one of the targets. Regarding cocaine intake, females acquire cocaine-SA sooner than males and are more prone to relapse compare to males (Algallal *et al.*, 2019). Therefore, in our conditions, I would not expect sex-specific alterations of the ECS.

ii. Are allosteric modulators more promising than orthosteric ligand?

CB1 antagonists are a highly interesting to treat addictive disorders, however, as stated previously, orthosteric blockade of CB1 triggers severe psychiatric side effects (Taylor, 2009). Therefore, another interesting strategy would be to reduce CB1 activity instead of blocking it. Allosteric modulators are interesting tools to induce such effects. Indeed, allosteric modulators do not modulate directly the activity of a GPCR but instead either enhance or inhibit an agonist binding affinity and signaling efficacy of ligands (Ross, 2007). In that context, a pretreatment with

ORG27569, a CB1 negative allosteric modulator, attenuated both cocaine and methamphetamine seeking in rats (Jing *et al.*, 2014). Furthermore, administration of pregnenolone, another CB1 negative allosteric modulator, reduced CB1 activity and also decreased WIN55.512-2-SA and motivation towards WIN55.512-2 intake in mice (Vallée *et al.*, 2014). Thus, these findings strongly suggest that allosteric modulators are promising tools to treat addictive behaviors. Giving the adverse effects elicited by CB1 antagonists, studying further their potential is a necessity.

More recently, RVD-hemopressin (α) (a hemoglobin α chain derived-peptides) has been proved as a CB1-negative allosteric modulator. Very interestingly, RVD-hemopressin (α) diminished food intake (Ferrante *et al.*, 2017) and even in a cafeteria diet paradigm (Leone *et al.*, 2018). As previously stated, this new line of strategy is very promising, however further investigations and clinical studies are needed to better assess their efficiency and safety in humans.

iii. Cell diversity: can we overcome this issue? What about astroglial or mitochondrial CB1? Microglial CB2?

A common issue with our type of research is that we cannot discriminate the alterations we observed from neurons or glial cells. As stated previously, CB1 is expressed in GABAergic, glutamatergic (Martín-García *et al.*, 2016) and astrocytes (Navarrete & Araque, 2008) whereas CB2 is expressed in dopaminergic neurons (Zhang *et al.*, 2017) and microglial cells (Atwood & Mackie, 2010). In structure like the striatum, cell heterogeneity is widely attenuated giving that 90-95% of all neurons are medium spiny neurons but still, there are at least 10 different cell types (neurons, glial, vascular, ependymal and stem cells) in striatum (Gokce *et al.*, 2016).

Fluorescence activated cell sorting can overcome this issue. Using this method, brain tissue is enzymatically and mechanically dissociated into single cells, which are then fluorescently labelled with antibodies targeting a specific cell type and forced to pass single-file through a narrow flow cell in a flow cytometer (Cruz *et al.*, 2013). Thus, we could target only CB1 or CB2-expressing cells using specific antibodies and separate neurons from glial cells. Similarly, fluorescence activated nuclear sorting is also a powerful method. For instance, Kozlenkov et al. were able to discriminate the DNAm profiles between glutamatergic and GABAergic neurons from postmortem human brain samples using specific nuclear markers of neurons (Neun+) and GABAergic (SOX6+) cells (Kozlenkov *et al.*, 2016).

In our project, we could easily discriminate the ECS alterations between GABAergic and glutamatergic neurons or even investigate only mitochondrial-CB1 expressing cells. Using such approaches, we could therefore better understand whether cocaine impacts the ECS on GABAergic or glutamatergic cells. This is an important issue as GABAergic or glutamatergic CB1

neurons modulate differently cocaine-SA (Martín-García *et al.*, 2016). This would bring clarity among our results as both GABAergic and glutamatergic CB1 neurons are expressed in the HPC. However, despite their appeal, these technologies are still very tricky to develop and exhibit other limitations such as limited number of samples.

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G. Annexes

I. Annex 1

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Review article

Neuroepigenetics and addictive behaviors: Where do we stand?

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ABSTRACT

Substance use disorders involve long-term changes in the brain that lead to compulsive drug seeking, craving, and a high probability of relapse. Recent findings have highlighted the role of epigenetic regulations in controlling chromatin access and regulation of gene expression following exposure to drugs of abuse. In the present review, we focus on data investigating genome-wide epigenetic modifications in the brain of addicted patients or in rodent models exposed to drugs of abuse, with a particular focus on DNA methylation and histone modifications associated with transcriptional studies. We highlight critical factors for epigenomic studies in addiction. We discuss new findings related to psychostimulants, alcohol, opiate, nicotine and cannabinoids. We examine the possible transmission of these changes across generations. We highlight developing tools, specifically those that allow investigation of structural reorganization of the chromatin. These have the potential to increase our understanding of alteration of chromatin architecture at gene regulatory regions. Neuroepigenetic mechanisms involved in addictive behaviors could explain persistent phenotypic effects of drugs and, in particular, vulnerability to relapse.

1. Introduction

Drug addiction or substance use disorder (SUD) is a complex brain disease involving long-term changes that lead to compulsive drug seeking, craving, and a high probability of relapse (Berridge, 2017; Volkow et al., 2016). Uncontrolled drug intake is associated with negative emotional states like anxiety when the drug is not available, and occurs despite individual and social negative consequences (Everitt and Robbins, 2005; Koob, 2009). The neuronal circuits involved in addictive behaviors are complex (Koob and Volkow, 2010), and imaging tools have identified disrupted brain structures in addicted humans that are implicated in addiction vulnerability (Volkow and Morales, 2015). Key structures include the mesolimbic dopaminergic system, with the ventral tegmental area (VTA) neurons projecting to the nucleus accumbens (NAc part of the ventral striatum). Other regions involved in motivational and emotional processes or in memory association such as the dorsal striatum (DS), prefrontal cortex (PFC), insula, extended amygdala and hippocampus (hipp), participate in this reward circuit. The rostromedial tegmental nucleus (RMTg) has been recently described to play a role in the control of the mesolimbic pathway (Bourdy and Barrot, 2012). Also, the lateral habenula is disrupted by drugs of abuse and proposed to be a hub in addictive responses (see Mathis & Kenny, in this special issue, and Velasquez et al., 2014). Only a proportion of individuals become addicted and this vulnerability, meaning the transition from recreational to uncontrolled and compulsive intake, is largely impacted by the nature of the drug, genetic factors, in addition to the developmental, social and the psychological context of the individual (EMCDDA, 2017; Juli and Juli, 2015; Kreek et al., 2005).

Knowledge of molecular mechanisms involved in behavioral adaptations observed following repeated drug exposure has greatly improved over the last decade. Modifications at the level of neuron connectivity signaling, dendritic spine morphology or synaptic plasticity (Robinson and Kolb, 1999; Ron and Jurd, 2005) have been described together with transcriptional regulations (reviewed in (Contet et al., 2004; McClung et al., 2005; Przewlocki, 2004; Rhodes and Crabbe, 2005; Russo et al., 2010; Spanagel and Heilig, 2005). Increasing evidence is now emerging that epigenetic modulations participate in

Abbreviations: 5hmC, 5-hydroxymethylcytosine; 5mC, 5-methylcytosine; CPP, conditioned place preference; DNAhm, DNA hydroxymethylation; DNAm, DNA methylation; DNMT, DNA methyltransferase; DS, dorsal striatum; HDAC, histone deacetylase; MeCP2, methylated DNA-binding protein 2; miR, microRNAs; NAc, nucleus accumbens; PFC, prefrontal cortex; SUD, substance use disorder

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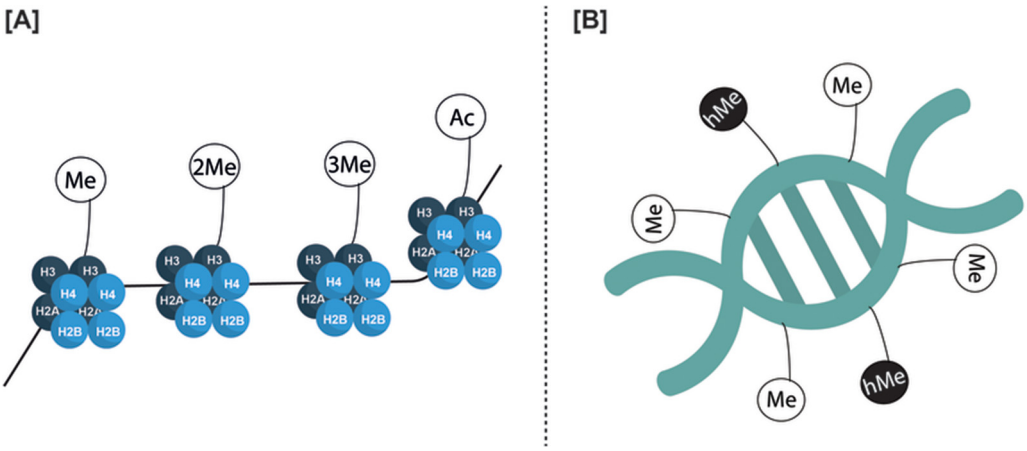

Fig. 1. A) Histone proteins (H2A, H2B, H3 and H4) form an octamer with two copies of each, which is wrapped around 147bp of DNA to form a functional unit of chromatin, the nucleosome. Modifications such as methylation (Me), bimethylation (2Me), trimethylation (3Me) or acetylation (Ac) can alter the N-terminal tails of these histones (here, H3 is depicted). These modifications lead to either an open (enable gene expression) or close chromatin state (repress gene expression). B) Addition of a methyl group to a cytosine (Me, position 5) can alter the DNA segment activity. When a promoter is highly methylated, this modification often leads to decrease gene expression. The methyl groups can be oxidized by the ten-eleven translocation enzymes family to form a hydroxymethyl group (hMe).

Table 1
Whole-genome epigenetic studies following psychostimulant exposure.

	Analysis	Species	Administration paradigm	Dose	Region	Reference
Cocaine	DNAm microarray	Rat	C, 10d i.v W, 1,30d	0.75 mg/kg	NAc	Massart et al. (2015)
	DNAm microarray	Mouse	A, i.p C, 8d C 7d, W 7d, A	20 mg/kg	NAc	Maze et al. (2010)
	MBD-seq	Mouse	C, 12d i.v W, 1,21d	0.5 mg/kg	mPFC	Baker-Andresen et al. (2015)
	MBD-seq	Rat	C, 13d i.v	0.33 mg/kg	mPFC	Fonteneau et al. (2017)
	5hmc-seq RNA-seq ChIp-seq	Rat	C, 7d i.p	20 mg/kg	NAc	Feng et al. (2015)
	RNA microarray	Human			Midbrain	Bannon et al. (2015)
	RNA microarray	Mouse	C, 7d e.o.d i.p	20 mg/kg	NAc	Bu et al. (2012)
	RNA microarray	Rat	C, 10d i.v W, 1,10,100d	1.5 mg/kg	NAc, mPFC	Freeman et al. (2010)
	RNA-seq	Mouse	C, 7d i.p	20 mg/kg	NAc, Striatal PSD	Eipper-Mains et al. (2011)
	RNA-seq	Mouse	C, 7d i.p W, 28d	20 mg/kg	NAc	Eipper-Mains et al. (2013)
	RNA-seq	Mouse	C, 8d e.o.d i.p E, 8d W, 8d R, i.p	5 mg/kg 1.25 mg/kg	NAc	Lo Iacono et al. (2016)
	RNA-seq	Mouse	C, 7d i.p	20 mg/kg	NAc	Wang et al. (2016b)
	RNA-seq	Mouse	C, 7d i.p	20 mg/kg	NAc	Cates et al. (2017)
	RNA-seq	Rat	C, 14d	0.5 mg/kg	NAc	Zhang et al. (2016)
	ChIp- ChIp	Mouse	C, 7d i.p	20 mg/kg	NAc	Renthal et al. (2009)
	ChIp-seq	Human			Hipp	Zhou et al. (2011)
	ChIp-seq RNA-seq	Human			Hipp	Farris et al. (2015)
	ChIp-seq	Mouse	C, 7d i.p	20 mg/kg	NAc	Maze et al. (2011)
	ChIp-seq	Mouse	C, 7d i.p	20 mg/kg	NAc	Ferguson et al. (2015)
	ChIp-seq	Mouse	C, 7d i.p	20 mg/kg	NAc	Damez-Werno et al. (2016)
	ChIp-seq RNA-seq	Mouse	C, 7d i.p	20 mg/kg	NAc	Feng et al. (2014); Hu et al. (2017)
	ChIp-seq	Mouse	C, 7d i.p	20 mg/kg	NAc	Sun et al. (2017)
	4C-seq	Rat	C, 7d i.p	20 mg/kg	NAc	Engmann et al. (2017)
Methamphetamine *	DNAm microarray	Mouse	C, 27d e.o.d i.p	0.5 to 4 mg/kg	Hipp	Itzhak et al. (2015)
	5hmc-seq	Rat	C, 20d e.o.d i.v	0.1 mg/kg	NAc	Cadet et al. (2017)
	RNA microarray	Rat	A, i.p	20 mg/kg	NAc	Martin et al. (2012)
	RNA-seq	Rat	C, 14d i.p W, 14d	1 mg/kg	PFC, OFC, NAc	Mychasiuk et al. (2013)
	RNA-seq	Mouse	C, 5d i.p W, 2d	2mg/kg challenge: 5 mg/kg	NAc	Zhu et al. (2015)
	ChIp-seq RNA microarray	Rat	A, i.p	2 x 5 mg/kg	Striatum	Cadet et al. (2013)
MDMA	RNA microarray	Mouse	C, 11d i.v	0.125 mg/kg	NAc, FC, dorsal raphe nucleus, Hipp	Fernandez-Castillo et al. (2012)

(*: transgenerational study; A: acute; C: chronic; W: withdrawal; E: extinction; R: reinstatement; e.o.d: every other day; i.p: intraperitoneal; i.v: intravenous; d: day; y: year).

vulnerability to addiction, as well as in the maintenance of behavioral adaptations induced by substance abuse. Epigenetic mechanisms involve chromatin modifications (chemical or conformational) that alter gene function without changing the DNA sequence. These mechanisms are often initial responses to a dynamic environment and regulate several processes including gene expression, DNA replication and repair, growth, cell cycle and, development. In the last years, such dynamic modifications have been specifically studied in neurons, in relationship to neurodevelopmental, psychiatric and neurodegenerative disorders (Cholewa-Waclaw et al., 2016; Francelle et al., 2017; Nestler et al., 2016). As a result, the concept of neuroepigenetics emerged to specifically describe epigenetic regulations occurring in post-mitotic neurons as opposed to epigenetic mechanisms involved in developmental processes (Day and Sweatt, 2010). Several neuroepigenetic regulations occur throughout reward circuitry to regulate gene expression changes following exposure to drugs of abuse (Walker et al., 2015), which include addition of epigenetic marks on histone proteins or DNA sequence (Fig. 1 and see below). Additional modifications involve changes in non-coding RNA levels that impact gene expression and function and have been recently described in cocaine addiction (Smith and Kenny, 2018). They do play a critical role in synaptic plasticity underlying addiction and will not be detailed here (Mayfield, 2017; Smith and Kenny, 2018). As the epigenetic field is rapidly evolving, recent reviews dedicated to specific drugs like alcohol (Farris et al., 2015; Pandey et al., 2017), cannabinoids (Szutorisz and Hurd, 2017) or cocaine (Sadri-Vakili, 2015; Vaillancourt et al., 2017; Zwiller, 2015), and to a more global view of addiction (Nestler, 2014) have been published to highlight the importance of epigenetic dysregulations in addictive behaviors.

In the present review, we focus our attention on recent data interrogating global epigenetic modifications (genome-wide) in the brain following drugs of abuse in humans, non-human primates or rodent models, with a particular focus on DNA methylation and histone modifications, associated with transcriptional studies. We specifically highlight critical factors that should be considered when evaluating neuroepigenetics reprogramming. We discuss whether shared mechanisms or biomarkers have been identified within epigenetic programming following drug exposure. We highlight how developing tools, including investigation of structural reorganization of the chromatin, will increase our understanding of these molecular adaptations in addictive behaviors, that could explain persistent phenotypic effects of drugs and, in particular, vulnerability to relapse.

2. Critical factors for epigenome analysis in addiction

Recent studies in the addiction field have focused on epigenomic approaches, with the analysis of global changes at the level of chromatin for DNA methylation or histone modifications. These data are listed for psychostimulants (Table 1), alcohol (Table 2), opiate (Table 3), nicotine or cannabinoid (Table 4), the latter still being scarce. Recent data are emerging from genome-wide analysis, using chromatin immunoprecipitation followed by massive parallel sequencing (ChIP-seq). This allows quantification of the amount of immunoprecipitated fragmented chromatin for all genomic regions by high-throughput sequencing, using an antibody specific for a given targeted histone mark. Also, for global analysis of epigenetic dysregulations, a concomitant analysis of the gene expression profile represents a powerful tool to correlate the observed adaptations. Transcriptional analysis within brain reward circuitry has been widely performed in the field of addiction using either candidate approaches or microarray technologies (for reviews see (Contet et al., 2004; McClung et al., 2005; Przewlocki, 2004; Rhodes and Crabbe, 2005; Russo et al., 2010; Spanagel and Heilig, 2005). The emergence of high-throughput sequencing with the development of RNA-seq allows powerful transcriptional profiling. This method, based on the creation of cDNA libraries from isolated RNA, provides a reliable analysis of the transcriptome, at a higher base-pair resolution than classical microarray, and allows detection of non-coding RNAs, or alternative splice variants (Box 1). We therefore also examined studies, which also provided transcriptomic analysis using RNA microarray or RNA-seq approaches, enabling to assess drug-induced epigenetic changes on gene expression programs. Specific results will be discussed below but several factors have to be considered when examining such genome-wide studies and we highlight the most critical ones here (Fig. 2).

2.1. Animal models

Most of the studies investigating modifications in the brain have been performed in rodents, with the exception of a few ethanol studies, that examined human or rhesus macaque samples (see Table 2). Also, we have not included studies investigating epigenetic adaptations from human blood samples. Using rodent animals, one expects to obtain less variability compared with human studies performed on large cohorts of persons with distinct and often unknown histories of addiction. In contrast, various behavioral responses to drugs of abuse, like alcohol, are also observed in animal models within a homogenous population. This vulnerability may be partly explained by subtle environmental changes that can also induce epigenetic modifications. Also, differences may be identified when comparing drug effect on distinct strains at the level of behavior (Ayranci et al., 2015) or transcriptomic adaptations (Grice et al., 2007; Korostynski et al., 2007). Still, rodent models are very useful to study drug effects under more controlled conditions.

2.2. Diversity of paradigms

Among the rodent studies, it is interesting to note that various protocols have been developed, from acute drug treatment (A) to subchronic or chronic (C) administrations and sometimes following withdrawal periods (W). Doses, route of administration as well as duration of treatments vary extensively across studies making it difficult to describe a global modification scheme. Also, most studies analyzed passive drug administration with very few studies investigating voluntary consumption in rodent models (see below, (Baker-Andresen et al., 2015; Cadet et al., 2017; Fernandez-Castillo et al., 2012; Fonteneau et al., 2017; Freeman et al., 2010; Massart et al., 2015). When more studies are available, it will be useful to compare passive versus voluntary intake to evaluate possible transcriptomic or epigenetic signatures of voluntary consumption of drugs of abuse.

In SUD, increasing research to investigate mechanisms that could explain long term adaptations leading to relapse have been developed. Nevertheless, the neurobiological mechanisms involved in long-term drug abstinence are still poorly understood. Very few studies investigating whole-genome changes have focused their attention on abstinent conditions. Earlier studies have investigated drug abstinence at the transcriptome level (Kuntz-Melcavage et al., 2009; Spijker et al., 2004) and even fewer studies directly compared adaptations across drugs. Interestingly, in an open-ended approach using microarray, chronic morphine treatment revealed a collection of genes in the extended amygdala which represented promising candidates potentially involved in drug dependence and craving (Befort et al., 2008). Expression of these candidate genes were evaluated following four weeks of cessation of drug treatment (morphine, nicotine, THC and alcohol) and a common transcriptional signature of protracted abstinence was identified (Le Merrer et al., 2012). Interestingly, unlike other drugs, cocaine differentially altered some behavioral responses in abstinent conditions and, oppositely modified expression of several genes (Becker et al., 2017). These findings emphasize differences between cocaine addiction and addiction to other drugs, though highlighting commonalities between opiate, alcohol, THC and nicotine abuse. These data complete accumulating evidence that specific adaptations in terms of behavior, connectivity, morphology or transcription can occur depending on the type of drug and questioned the unitary theory of

Table 2
Whole-genome epigenetic studies following alcohol exposure.

		Analysis	Species	Administration paradigm	Dose	Region	Reference
Alcohol		DNAm microarray	Human			FC	Manzardo et al. (2012)
		DNAm microarray	Human			Precuneus	Hagerty et al. (2016)
		DNAm microarray	Human			PFC	Wang et al. (2016a)
		Bis-seq	Rhesus macaques	C, 1y oral	4%	NAc	Cervera-Juanes et al. (2017a,b)
	*	Bis-seq	Rat	2cycles of C, 3d + 3d oral W, 2d	20%	Hypo	Asimes et al. (2017)
		RNA microarray	Human			PFC	Zhang et al. (2014)
		RNA-seq	Human			Hipp	Enoch et al. (2013), (2014)
		RNA-seq	Mouse	C, 60d e.o.d oral	15%	Microglial cells	McCarthy et al. (2018)
		RNA-seq	Rat	C, 56d oral C, 28d oral + 21d gavage	6% 6% + 30%	NAc	Morud et al. (2017)
		RNA-seq	Rhesus macaques	C, 1y oral	4%	CeA	Iancu et al. (2018)
		RNA-seq	Mouse	4 cycles of C, 4d inhalation W, 2d C, 5d oral	inhalation 16 h/d 15%	NAc, Striatum, BNST, Amy, VTA, PVC	Mulligan et al. (2017)
	*	RNA-seq RNA microarray	Rat	A: 2 in. at GD12 i.p	2.9 g/kg + 1.45 g/kg	Amy, NAc	Ignacio et al. (2014)
		RNA microarray	Rat	PD4 to PD9 2 in./d oral	2.625 g/kg	Hipp	Balaraman et al. (2017)
		RNA microarray	Mouse	A, i.p	1.8-g/kg	Hipp	Baker et al. (2017)
		RNA microarray	Mouse	A, PD4&7 2 in./d s.c	2 x 2.5 g/kg	Hipp	Chater-Diehl et al. (2016)
	*	RNA microarray	Mouse	C, 8d oral	10%	Hipp	Marjonen et al. (2015)
	*	RNA microarray	Rat	A, 2 in. at GD12 i.p	2.9 g/kg + 1.45 g/kg	Amy, NAc	Middleton et al. (2012)
		RNA-seq	Mouse	*Selective breeding of mice for ethanol preference*		NAc	Colville et al. (2017)
		RNA-seq	Rat	*Predisposition for ethanol preference*		Whole brain	Saba et al. (2015)

(*: transgenerational study; A: acute; C: chronic; W: withdrawal; E: extinction; R: reinstatement; e.o.d: every other day; i.p: intraperitoneal; i.v: intravenous; d: day; y: year; PD: postnatal day; GD: gestational day; CeA: central amygdala).

addiction proposing common mechanisms for dependence and relapse processes (Badiani et al., 2011; Ozburn et al., 2015). Altogether, while whole-genome approaches for epigenetic adaptations are still needed to explore this hypothesis, one has to keep in mind that generalizing conclusions from these studies mostly obtained with psychostimulants may not be always pertinent for the other drugs of abuse.

In summary, time-dependent effects of drugs of abuse on transcriptional and epigenetic modifications represent a critical factor, as emotional deficits are still detectable after long periods of withdrawal (Goeldner et al., 2011). Several time-points should be included in future studies, and as proposed by Baker-Andersen (Baker-Andresen et al., 2015), including animals with a history of drug seeking will bring insight into the memory process altered by drug exposure. This will be particularly important to decipher specific mechanisms involved in states facilitating relapse vulnerability. In addition, the adolescent period represents a time when drug exposure increases the risk of addiction. There is limited research examining epigenetic changes in normal brain maturation during adolescence (Mychasiuk and Metz, 2016), but these data will further our understanding of these complex processes for a more complete comprehension of how the effects of drug exposure changes across time.

2.3. Brain structure or cell type targets

The complexity of the reward-circuit makes the comparison across studies challenging as targeted brain structures vary across studies. Interestingly, most epigenetic studies focused on the NAc and PFC (see Tables), which represent the structures where most initial transcriptomic studies were performed (Nestler, 2014). Among key reward-related structures, the NAc has been widely studied as the motivational center in reward related responses and is a critical structure for the initial rewarding effects of psychostimulants, whereas the PFC was

Table 3
Whole-genome epigenetic studies following opiate exposure.

		Analysis	Species	Administration paradigm	Dose	Region	Reference
Morphine		RNA microarray	Mouse	A, i.p C, 5d ; 3/d i.p	20 mg/kg 10,20,40 mg/kg	Striatum	Korostynski et al. (2007)
		RNA microarray	Mouse	C, 5d s.c	25 mg pellet	NAc	Grice et al. (2007)
		RNA microarray	Mouse	C, i.p 6d	20-100 mg/kg	Extended amygdala	Befort et al. (2008)
		RNA microarray	Mouse	S, 6 h s.c C, 4d s.c	25 mg pellet	Hypo	Anghel et al. (2010)
	*	RNA-seq	Rat	C, 21d i.v E, 10d R, i.p	0.25,0.75,1.25 mg/kg 1 mg/kg	NAc	Vassoler et al. (2017)
Heroine	*	DNAm microarray	Human			DLPFC	Chatterton et al. (2017)
		DNAm microarray	Human			OFC	Kozlenkov et al. (2017)
		RNA microarray	Human			NAc	Egervari et al. (2017)
		RNA microarray	Rat	C, 7d i.p	1 mg/kg	NAc	Yan et al. (2017)
		RNA-seq	Rat	C, 19d i.v E,1d	0.06 mg/kg-19d	NAc	Imperio et al. (2016)

(*: transgenerational study; A: acute; C: chronic; W: withdrawal; E: extinction; R: reinstatement; e.o.d: every other day; i.p: intraperitoneal; i.v: intravenous; d: day; y: year;).

Table 4
Whole-genome epigenetic studies following cannabinoid or nicotine exposure.

		Analysis	Species	Administration paradigm	Dose	Region	Reference
THC	*	Bis-seq	Rat	C, PD28 to PD49 e.o.3d i.p	1.5 mg/kg	NAc	Watson et al. (2015)
		RNA microarray	Mouse	C, 4.5d i.p	10 mg/kg	Cerebellum	Colombo et al. (2009)
Nicotine	*	RNA microarray	Mouse	C, 21d oral W, 90d	200 µg/ml	Cerebral cortex	Jung et al., (2016)
		RNA-seq	Human			DLFC	Tao et al. (2017)
		RNA-seq	Mouse	C, 14d s.c	2mg/kg/h mini osmotic pumps	SNc neurons	Henley et al. (2013)
	*	RNA-seq	Mouse	C, 5d by inhalation	13-16 mg/ml	FC	Lauterstein et al. (2016)
	*	RNA-seq	Mouse	C, 28d oral	200 µg/ml	*Pomc* neurons	Silva et al. (2016)
		RNA-seq	Rat	C, 14d i.p	0.3 mg/kg	PFC, OFC, NAc	Mychasiuk et al. (2013)

(*: transgenerational study; A: acute; C: chronic; W: withdrawal; E: extinction; R: reinstatement; e.o.d: every other day; i.p: intraperitoneal; i.v: intravenous; d: day; y: year; PD: postnatal day).

investigated, primarily, for its role in goal-directed behavior and decision-making. Adaptations within the dorsal striatum (DS) have received far less attention than those occurring in the NAc, while changes in this structure would better inform epigenetic regulations in link with expression of compulsive drug-seeking traits, including relapse (Belin and Everitt, 2008). Brown and colleagues showed decreased expression of synaptic plasticity-associated genes in the DS of animals categorized as relapse-vulnerable (Brown et al., 2011), a regulation related to decreased expression of specific miRNA in subregions of the DS (Quinn et al., 2015). It seems critical to evaluate changes in homogenous structures with few different cell types as there is now accumulating evidence that epigenetic modifications may be specific to particular brain structures and even to cell-types. Even subtypes of medium spiny neurons of the NAc are differently affected by epigenetic modifications (Hamilton et al., 2018). Several techniques have been developed to enrich or isolate specific cells from brain tissue, including immunopanning, laser capture micro-dissection, fluorescence-activated (FAC) sorting and magnetically labeled antibodies (see Holt and Olsen, 2016). The latter technique allows neuronal, astrocytic, and microglia cell populations to be sorted in adult rodent brain at a low cost. Distinguishing between neurons and glial cells, as recently performed using FAC sorting in human samples of heroin addicts, appears now to

Box 1
Tools for transcriptomic and epigenomic studies.

Transcriptomic profiling (see (Hitzemann et al., 2013; Wang et al., 2009)

***RT-qPCR*,** gene expression analysis for candidate genes

RNA microarrays, initial genome-wide gene expression profiling using oligonucleotide arrays and hybridization-based approaches.

RNA-Seq, a high throughput sequence-based method. RNA are converted into libraries of cDNA and each molecule is further sequenced with read between 30–400 bp. Reads are then aligned to a reference genome. It allows to analyze alternative splicing, the expression of coding and non-coding RNAs with a higher base-pair resolution

DNA methylation profiling see (Kurdyukov and Bullock, 2016)

***Whole-genome methylation*,** approaches to quantify the amount of deoxycytidine (dC) and methylated cytosines (5mC) in a hydrolysed DNA sample using high performance liquid chromatography (ultraviolet), liquid chromatography coupled with mass spectrometry, pyrosequencing LINE-1. These methods do no offer a precise quantification of methylation levels.

***Bisulfite sequencing*,** conversion by bisulfite of cytosine into uracil while 5-methylcytosines are not affected. Following conversion, either whole genome bisulfite sequencing or reduced representation bisulfite sequencing where CpG regions are enriched can be processed.

Methylcytosine binding proteins (MBD) and antibodies targeting 5mC (MeDIP), approaches allowing enrichment of differentially methylated regions (DMR), can be performed before bisulfite treatment.

***DNAm microarrays*,** following bisulfite conversion, allows using specific microarrays to interrogate region-methylation levels such as promoters or gene bodies.

***DNA digestion*,** particular endonucleases are able to digest CCGG sequence to analyze either whole-genome or specific gene methylation levels.

Chromatin profiling see (Park, 2009)

ChIp, Chromatin immunoprecipitation, state of the art technique to study chromatin alterations such as DNA-binding proteins, histone modifications and nucleosomes alterations.

***qChIP*,** investigats a single locus by qPCR following chromatin immunoprecipitation.

***ChIP-ChIP*,** interrogates selected-region modifications using DNA microarrays.

***ChIP-Seq*,** whole-genome sequencing following chromatin precipitation with high base-pair resolution.

DamID-Seq, indentification of DNA adenine methyltransferase without immunoprecipitation step, based on a fusion protein and E. coli DNA adenine methyltransferase; limited to *in vitro* models.

***ATAC-Seq / DNA-Seq*,** addresses chromatin accessibility, providing similar information to RNA-seq but focusing on DNA; used for profiling enhancer regions.

Chromosome conformation capture (see (Davies et al., 2017; Dekker et al., 2017)

3C, analysis of interactions between a single pair of genomic loci.

4C, identification of interactions between one locus and all other genomic loci.

5C, study of functional contacts between all the genes within a given region.

Hi-C, identification of functional contacts through all parts of the genome.

Imaging techniques, mapping of chromatin interactions.

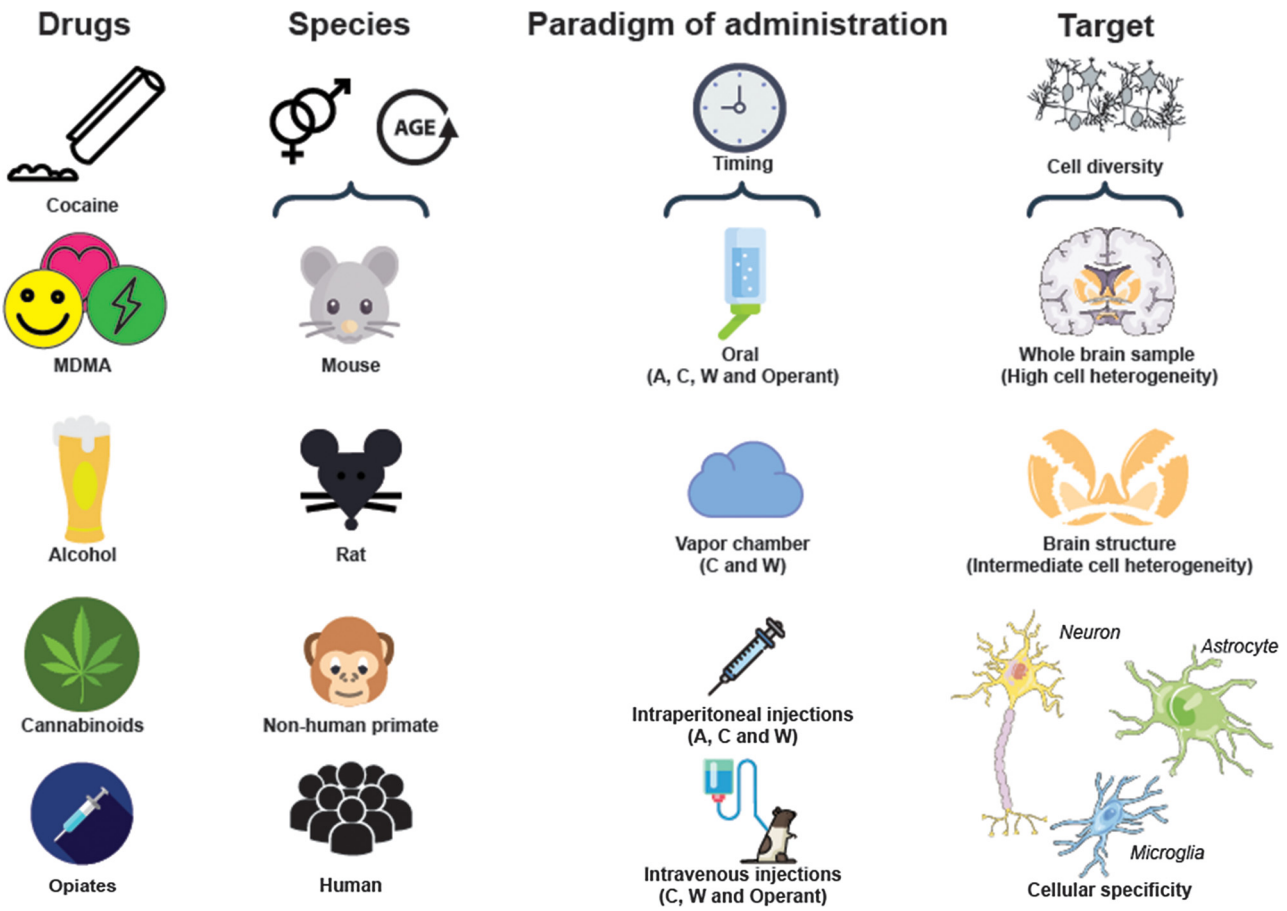

Fig. 2. Representative scheme illustrating some critical factors for the analysis of neuroepigenetic modifications by drugs of abuse (cocaine, MDMA, alcohol, cannabinoids and opiates). Species, sex and age of the individuals can modify observed effects. Several paradigms of drug administration are classically used, with distinct timing (acute, chronic, abstinent) to study direct drug effects or relapse, various modes of administration (oral, vapor, intraperitoneal or intravenous), with passive or voluntary access to the drugs (choice or operant system). They all enable to study addiction-related behavioral responses. The genome-wide analysis can be performed on whole brain or microdissected samples to focus on specific brain structures. Recent technologies allow now to lower cellular heterogeneity and focus on specific cell types. A: acute, C: chronic, W: withdrawal.

be critical for a deep analysis of epigenetic mechanisms (see below, §3.2 (Kozlenkov et al., 2017). Noteworthy, few whole-genome studies directly compared transcriptomic changes occurring across several structures following nicotine and methamphetamine (Mychasiuk et al., 2013) or alcohol (Mulligan et al., 2017) exposure (see Tables 1–3), but no specific or global epigenetic signatures were established.

2.4. Sex differences

Risk taking is often higher in males, but effects of many drugs appear more deleterious in females. Indeed, anxiety and depression are higher in females and these represent strong predictors of addiction. One study explored epigenetic changes in both sexes for DNAm following ethanol exposure in the human cortex (Wang et al., 2016a). Interestingly, no adaptations could be observed in females. Whether this was due to a low number of female individuals under study or really reflects a sex difference is not clear. Interestingly, the study by Engmann on 3D chromatin conformation showed a specific increase in *Auts2* gene only in male mice (see below § 6.1, (Engmann et al., 2017). More studies are needed to explore these aspects to better understand epigenetic mechanisms in the future and clarify how gene expression influences individual heterogeneity in vulnerability to addiction.

3. Changes in DNA methylation and hydroxymethylation

DNA methylation (DNAm) and hydroxymethylation (DNAhm) have been predominantly studied in the mammalian developing brain and described as dynamic processes, as they play a critical role in the establishment and maintenance of cell identity (Bogdanovic and Lister, 2017). DNA methylation corresponds to the addition of a methyl group, usually on the 5′ position of the carbon of the pyrimidine ring of cytosine, and occurs at dinucleotides CpG and CpH (H = A/C/T) (see Fig. 1) (Kinde et al., 2015; Lister et al., 2013). This modification (5-mC), when occurring in the promoter region, is mostly associated with transcriptional repression and is catalyzed by DNA methyltransferases involved in the maintenance of methylation (DNMT1) or in de novo methylation (DNMT3 a and b). Demethylation processes are conducted by ten-eleven translocation enzyme which catalyzes the hydroxylation of the methylated cytosine (5hmC). Other forms are also produced, with 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which are processed by DNA repair mechanisms and further generate unmethylated cytosine (Auclair and Weber, 2012). In contrast, hydroxymethylation in intragenic regions is associated with higher gene expression (Guibert and Weber, 2013; Kato and Iwamoto, 2014). Proteins with methylated DNA binding domain (MBD) play a critical role in DNA methylation, with the protein methyl CpG-binding protein 2 (MeCP2) being one critical reader of DNA methylation in the brain (Kinde et al., 2015). MeCP2 protein level is increased by cocaine and contributes to behavioral responses to psychostimulants (Cassel et al., 2006; Deng et al., 2010; Im et al., 2010). However, additional studies are needed to better understand the implications of this process. Methods for comprehensive analysis of DNA methylation and hydroxymethylation include bisulfite

sequencing, or collection of methylated, hydroxymethylated, or unmethylated DNA by specific binding proteins, antibodies, or restriction enzymes, followed by sequencing or microarray analysis. These techniques allow measurement of DNA methylation at a single-base resolution (see Box 1).

3.1. Correlation of DNAm with transcriptional adaptations

The possible correlation of DNAm changes induced by different drugs of abuse and gene expression at a genome-wide scale has been examined in a number of studies. In one, psychostimulant exposure induced differentially methylated regulations which were correlated with gene expression adaptations. In a study examining the effect of cocaine self-administration, combination of whole-genome and candidate approaches allowed identifying DNAm regulations in the NAc, which were partly negatively correlated with gene expression changes (Massart et al., 2015). Analysis of 5hmC (hMeDIP-Seq) in the NAc following a model of compulsive methamphetamine self-administration showed similar correlation (Cadet et al., 2017). Hydroxymethylation peak changes were found in intergenic sites while only few modifications were present at transcription start sites, exons and, introns. Interestingly, potassium channel genes with increased DNA hydroxymethylation peaks at intergenic sites were associated with an increased expression of potassium channel coding genes in exposed but non-compulsive rats (Cadet et al., 2017). In another study using methylome analysis in the PFC following self-administration of cocaine in rats, the authors have revealed that increased DNAm were inversely correlated to transcriptional activation when methylation occurred in the gene promoter. Interestingly, this was not observed when methylation took place inside gene bodies (Fonteneau et al., 2017), where a strong variation was observed in the tested genes with both up- or down- regulations associated with hypermethylation. Alternative promoters in gene bodies or the presence of alternative splice sites could explain this observation. Also, similar methylation levels can occur in active and inactive gene bodies across brain tissue (Aran et al., 2011). A better understanding of these modifications will be provided with a more complete genome annotation and improved description of alternative promotor or splice sites. Others have described more mixed results in a cocaine self-administration paradigm, with both validation of transcriptional regulation associated with DNAm and non-regulated transcripts (Baker-Andresen et al., 2015). Whether these correlations are specific to the type of drugs is not clear. Indeed, following alcohol exposure, Wang et al. investigated DNAm changes in PFC postmortem samples from individuals with alcohol use disorders (Wang et al., 2016a). They found 1812 differentially methylated CpGs among which the majority were hypermethylated (1201 CpGs), and preferentially found in promotor regions and gene bodies. When analyzing these data together with previous transcriptomic data from the same samples (Zhang et al., 2014), the authors did not observe any correlation between DNAm changes and gene expression. The authors argue that this could be due to single nucleotide polymorphism on DNAm affecting gene expression. A similar finding with no correlation between DNAm and gene expression was obtained in a transgenerational study in rats using a binge alcohol protocol at adolescence (see §5.2, (Asimes et al., 2017). One can-not exclude the fact that the complexity of data in genome-wide analysis and the accuracy of genome annotation make bioinformatic analysis difficult for such correlations. Also, double analysis of DNAm and hydroxymethylation in future studies may help to clarify these aspects. Together, these data indicate more complex mechanisms for DNAm processes than initially described, which may specifically differ depending on the methylated gene loci or the drug abused.

3.2. Cellular specificity of DNAm

In a global DNA methylation profiling (DNAm microarray) of orbito frontal cortex (OFC) from heroin addicts who died of overdose, the authors investigated heroin effects on DNAm specifically in neuronal nuclei separated by FAC sorting (Kozlenkov et al., 2017). Hypermethylated regions were preferentially found in exons and gene bodies and mostly depleted from promoter regions, in genes involved in synaptic plasticity. In contrast, hypomethylated regions were preferentially found in promoter regions. To complete their analysis, they performed H3K27Ac ChIP-seq and showed that only hypomethylated regions were enriched in enhancers, at both distal (putative active enhancers) and proximal (active promoters) regions from the transcription start site, while hypermethylated regions were depleted. Investigating modification specifically in neurons reveals enhanced hypermethylated gene regions in genes enriched preferentially in glutamatergic, but not GABAergic, neurons. Altogether, these results are in accordance with reduced glutamatergic transmission in the frontal cortex observed following drug exposure and particularly highlight that DNA methylation changes in neurons are specific to targeted gene regions. Other studies have recently analyzed transcriptomic profiling using RNA-seq in targeted cell-types. These studies were conducted in rodent paradigms with prolonged nicotine exposure, using laser dissected *Pomc* neurons, known to be involved in nicotine-anorectic effects (Silva et al., 2016), or SNc neurons, implicated in Parkinson disease and expressing high levels of nicotinic acetylcholine receptors (Henley et al., 2013). A recent study targeted microglial cells isolated from the prefrontal cortex of alcohol exposed mice, a population of cells involved in the neuroinflammatory response of alcohol (McCarthy et al., 2018). The results revealed subtle changes in gene expression following nicotine and a distinctive microglial gene expression signature for neuroimmune responses related to alcohol consumption. Targeting specific cell-types demonstrated the critical importance of studying DNAm changes in a highly homogenous cell population. Altogether, further studies dissecting cell-type specificity for epigenetic regulations should focus on more precise mechanisms involved in adaptations following drug abuse. Such approaches may encounter difficulties due to limitation in sorting cell-types or in DNA amount needed from specific brain structures. Nevertheless, technical advances are emerging very rapidly with approaches conducted with limited DNA amount or performing single-cell epigenome sequencing (Farlik et al., 2015; Wen and Tang, 2018), which will surely open more precise investigation of these neuroadaptations.

3.3. Dynamics of DNAm changes

Few studies have investigated the dynamics of DNA modifications at the transcriptomic or epigenetic levels. In the NAc of rats self-administering cocaine over 10 days, DNAm changes were examined either a day following the treatment (short) or 3 weeks after (prolonged withdrawal). The results revealed stable changes in DNAm at the two timepoints illustrating a persistent adaptation. Interestingly, several modifications were time-dependent, indicating a more dynamic process associated with DNAm in link with behavioral adaptations induced by cocaine (Massart et al., 2015). Using a similar cocaine self-administration model in rats, others have compared gene expression profiles (RNA microarray) following 1, 10 or 100 days of withdrawal (Freeman et al., 2010). Their analysis also revealed complex adaptations, with several categories of time-dependent changes in gene expression in NAc and PFC, including persistent or unstable changes throughout withdrawal. In an elegant study investigating the effect of cocaine at several steps of dependence in a mouse model of self-administration, the authors questioned the implication of adaptations from simple drug exposure versus learned cocaine-seeking (Baker-Andresen et al., 2015). The authors used methyl-binding protein immunoprecipitation followed by high throughput sequencing (MBD-seq) to measure DNAm in isolated neurons of mouse PFC. DNAm patterns were compared after acute and prolonged withdrawal or in a situation of relapse. Passive cocaine exposure and cocaine self-administration produced distinct

patterns of 5mC enrichment. Persistent methylations were observed across the time-points, mostly hypermethylation, which were embedded within genes or located distal. Hypermethylation induced by cocaine is in line with increased expression of *DNMT3A* and *3B* observed following repeated administration (Anier et al., 2010; LaPlant et al., 2010; Pol Bodetto et al., 2013). Also, some methylation profiles were specific to mice subjected to 3 weeks of abstinence following cocaine self-administration but not in animals following one day of withdrawal or in yoked cocaine controls (Baker-Andresen et al., 2015). These data highlight the complexity of dynamic changes in DNAm following drug exposure, with implications of changes related to withdrawal or to learning processes with the maintenance of cocaine-related memories. Further studies investigating such time-dependent changes in DNAm are needed to clarify epigenetic adaptations underlying the cognitive adaptations that lead to addictive behaviors. In particular, exploring drug-seeking and relapse represent a major challenge to understanding long-term process observed in human addiction. Whether these adaptations are specific to psychostimulants or share common mechanisms across drugs is still an open question. In addition to methylation, hydroxymethylation also plays a major role in this dynamic process (Bachman et al., 2014), and this adds an additional level of complexity to DNAm mechanisms in the context of SUD.

3.4. DNAm at splicing sites

Recent studies have highlighted that DNAm may occur at specific splicing sites in the gene and therefore not directly impact gene expression levels, but rather the expression of distinct variants. Consequently, this may differentially modulate synaptic plasticity or signaling process and thus have specific phenotypic impacts. In the study by (Baker-Andresen et al., 2015) (see above), 12 of 15 persistent gene-associated DMRs were located within intronic regions or non-coding loci, whereas principal promoter regions and exons were relatively devoid of changes. These DNAm changes induced by cocaine self-administration were able to modify alternative splicing and therefore expression of isoforms. Therefore, they demonstrated that the absence of an overall change in a gene expression did not exclude alteration of splice variant expression. In Nestler's group, using ChIP and RNA-seq in the NAc of cocaine-treated mice, they established chromatin and transcriptional profiles with the identification, for the first time, of the transcription factor *E2F3* as a regulator of cocaine-induced gene expression and alternative splicing (Feng et al., 2014). In a follow up experiment, they assayed the effects of NAc overexpression of *E2F3* isoforms on cocaine behavioral response and transcriptomic profiling (RNA-seq) (Cates et al., 2017). Interestingly, overexpression of *E2F3a* was sufficient to mediate cocaine-induced locomotor sensitization and cocaine CPP, and the knockdown prevented these responses. *E2F3a* overexpression induced differentially expressed genes and differential alternative splicing events in a similar manner as cocaine, and both cocaine and *E2F3a* overexpression increased *E2F3* binding at consensus sequences near alternative splicing sites (Cates et al., 2017). These findings reveal a crucial role for *E2F3a* as a key regulator of cocaine-elicited molecular actions with both transcriptional and splicing profile changes leading to behavioral adaptations to drugs of abuse. In a study analyzing previously published data using an innovative bioinformatics approach to model association between splicing sites and histone marks (Feng et al., 2014), Hu et al found two marks (H3K36me3 and H3K4me1) with the strongest association with alternative splicing (Hu et al., 2017), indicating a major role for histone methylation in this process. In another study from the same group, repeated cocaine administration effects on DNA hydroxymethylation (5hmC-seq) in the same brain structure revealed a majority of 5hmC distributed in gene bodies and intergenic regions (Feng et al., 2015). Combining these results with analysis of both ChIP-seq (H3K27Ac and H3K4Me1) and RNA-seq, the authors observed 24 genes showing an increase in both 5hmC and mRNA 24 h after the last cocaine injection. They demonstrated that 5hmC dynamic modulation correlated with putative enhancers. Moreover, 5hmC regulations were found at exon boundaries which indicated the involvement of 5hmC at splicing sites. Consequently, hydroxymethylation correlated with splicing isoforms up-regulation. Together, these results indicate that 5hmC alterations represent a mechanism inducing long lasting changes and play a major role in cocaine-induced adaptations. This may potentially be extended to other drugs of abuse.

Interestingly, altered splicing sites have been studied in neurodevelopmental disorders such as schizophrenia or depression. For example, an alternative splicing of *GAD1* encoding glutamic acid decarboxylase was altered by DNA methylation at a CpG island close to the putative promoter of *GAD1* in cortex and hippocampus of patients with schizophrenia (Tao et al., 2017). Whether this adaptation was specific to neurons is still not clear. Another study revealed potential alteration of splicing sites for the glutamate ionotropic kainate receptor *GRIK2* induced by hypomethylation at intronic sites in isolated astrocytes cells from PFC of individuals who died during a depressive episode (Nagy et al., 2015). Taken together, it underlines possible general mechanisms involved in psychiatric disorders, that would be common with addiction.

In summary, a combined analysis of methylome (both methylation and hydroxymethylation) and transcriptome will be informative in assessing splice variants expression and understanding time-dependent methylation changes following drug exposure. The current studies have highlighted a dynamic process of methylation and hydroxymethylation following psychostimulant exposure. Whether similar conclusion can be proposed for the other drugs of abuse has still not been explored. The human studies are confronted with the constraint of analysis of DNAm at a unique time point in postmortem tissue (Cecil et al., 2016b). Whether extrapolation of data from studies examining DNA methylation in blood samples would be feasible is still questionable.

4. Modifications of histones

Another epigenetic mechanism that influences chromatin structure and thus the interaction between DNA and histones is the direct occurrence of modifications at the level of the N-terminal part of the histone tail (Walker et al., 2015). For example, acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation modulate in a reversible manner the degree of compaction of the chromatin. Specific enzymes are implicated in these processes to establish the marks (writers) or erase them (erasers). Histone acetyltransferases and histone deacetylases (HDAC) target specific histone residues for acetylation and deacetylation, respectively. Histone methyltransferases and demethylase act in a similar fashion for methyl groups. This represents a dynamic process. Typically, amino-acid acetylation promotes an open chromatin state associated with transcriptional activation. Histone methylations, usually on Lys and Arg, lead to activation or repression depending on the targeted residue. Methylated Lys 9 of Histone 3 (H3K9me) and H3K27me are associated with transcription repression, while H3K4me enhances transcription. In addition, the location of these modifications, at the promoter or regulatory regions, increases the complexity level of these regulations. These posttranslational mechanisms in response to psychostimulants have been widely studied using candidate gene approaches (Rogge and Wood, 2013; Sadri-Vakili, 2015; Walker et al., 2015). In addition, new tools allow studying the chromatin accessibility with DNaseI-Seq/ATAC-Seq to profile open chromatin or the spatial organization with chromosome conformation capture (3C)-based techniques (see Box 1). Studies on DNAm and hydroxymethylation pointed to modifications at the level of enhancers, suggesting that their activity may be modulated by drugs of abuse by DNA methylation processes. Studying modifications of histones, in particular the ones associated with enhancers, will provide insights into the role of DNA methylation.

4.1. Histone acetylations

Most studies examining histone modifications focused on the effects of passive cocaine exposure (see Table 1). A first study from Nestler's group has examined the role of histone modification in addictive behaviors and demonstrated that chronic cocaine treatment induced histone deacetylase 5 (*HDAC5*) gene expression in the NAc (Renthal et al., 2007). This enzyme usually represses gene expression by deacetylating histones. Interestingly, overexpression of *HDAC5* in the NAc attenuated cocaine CPP while *HDAC5* deficient mice showed a behavioral enhancement. This epigenetic enzyme has also been implicated in methamphetamine craving in the DS (Li et al., 2017, 2015). The genomic effect of repeated cocaine in the NAc was further studied with chromatin immunoprecipitation coupled with promoter microarray analysis (ChIP-chip) for acetylated and methylated histone (Renthal et al., 2009). Cocaine increased both acetylation and methylation at many more genes than it induced decreases. There was no systematic correlation between chromatin modifications and gene expression. The results also revealed concomitant hyperacetylation of H3 and H4 in very few genes, suggesting that the two marks play distinct roles (Renthal et al., 2009). The authors used ChIP for ΔFosB to identify direct targets for this transcription factor known to be regulated by cocaine (Renthal et al., 2009). They identified a particularly interesting gene family, the sirtuins or Sirt (silent information regulator of transcription) which are histone deacetylases whose function in the nervous system is still not clearly understood. The authors identified a significant enrichment of the transcription factor on the *Sirt2* promoter together with an increase of acetylated H3, associated with an increase of *Sirt2* transcript in the NAc. The same finding was obtained for *Sirt1*. Also, only chronic cocaine was able to increase both *Sirt1* and *Sirt2* activity in the NAc, compared to a single drug administration. Interestingly, pharmacological activation and inhibition of sirtuins were able to respectively enhance and diminish cocaine CPP as well as electrical excitability of NAc neurons (Renthal et al., 2009). In addition, a dramatic reduction in cocaine self-administration was observed following inhibition of sirtuins. Altogether, these findings implicate sirtuins in regulating behavioral effects of cocaine but whether this effect is due to histone modification or to sirt modulation of signaling process is not fully established. With a similar protocol in mice, a ChIp-seq approach was performed and the authors demonstrated that *Sirt1* activation induced the transcription factor forkhead box O3 in the NAc. Interestingly, the overexpression of this factor in the NAc enhanced cocaine CPP (Ferguson et al., 2015). Altogether, these studies highlighted a role for sirtuins in cocaine-induced adaptations at the molecular and behavioral levels but the precise mechanism involved is still not fully established. Interestingly, *Sirt1* overexpression in the NAc increased cocaine or morphine rewarding effects (Ferguson et al., 2013), suggesting a role for the sirtuin family across drugs of abuse. Whether similar implication of these HDAC is also observed in self-administration paradigm would be critical to better understand underlying adaptations for long term drug exposure and relapse events. Noticeably, sirtuins have been implicated in depressive-like state, a disorder often associated with SUD (Kim et al., 2016).

Interestingly, histone acetylation in the striatum was also observed in a model of methamphetamine exposure using a combination of microarray and ChIP-seq analysis (Cadet et al., 2013). Particularly, methamphetamine caused significant increases in H4K5Ac binding which correlated with levels of gene expression. Many regulated pathways were identified such as cell death and survival and nucleic acid metabolism. As in Martin et al. (Martin et al., 2012), the authors found an upregulation of several transcription factors like *cFos*, *Egr1* and *Egr2*. In addition, their findings revealed that acute and chronic administration of methamphetamine induced differential regulations in striatal gene expression, with more increased gene expression after an acute injection and mostly decreased gene expression following chronic treatment. Also, acute treatment induced additional H4K5Ac binding sites in more genes than in the chronic situation. These observations suggest that epigenetic adaptations to single drug exposure may be distinct from adaptions to long-term exposure, which may have triggered other epigenetic factors to differentially impact gene expression. This highlights potential specific epigenetic modifications in situation of chronic treatment with psychostimulant and, moreover, in conditions involving drug-seeking associated learning process.

4.2. Histone methylations

Histone methylations seem to play a major role in cocaine effects, with activation or repression of gene expression depending on location and number of methyl groups involved. Nestler's group investigated the relationship between two histone lysine dimethyltransferase G9a and GLP (G9a-like protein) and cocaine treatment. The authors observed a down regulation of both proteins in the NAc, induced by chronic cocaine exposure, which was associated with a decrease of the repressive mark H3K9me2 (Maze et al., 2010). Using genetic animal model and viral approaches, the authors demonstrated that G9a down regulation was able to modify neuron morphology in the NAc and enhance preference for cocaine. However, G9a overexpression in the NAc shell of rats increased H3K9me2, which surprisingly enhanced motivation for cocaine self-administration associated with increased anxiety (Anderson et al., 2018). Repeated cocaine decreased both H3K9me2 and G9a in the NAc of mice and, infusions of an HDAC inhibitor increased global levels of histone acetylation but also, of repressive histone methylation and G9A expression, illustrating cross talk among different types of histone modifications in the adult brain (Kennedy et al., 2013). Also, in a study modulating G9a expression specifically in *Drd1* or *Drd2* expressing neurons using genetic models or overexpression, distinct behavioral effects in response to cocaine were highlighted (Maze et al., 2014). In particular, selective deletion of G9a in *Drd2* neurons resulted in the unsilencing of transcriptional programs normally specific to *Drd1* neurons, coupled with acquisition of *Drd1*-associated projection and electrophysiological properties. Therefore, the authors proposed a new role for G9a in contributing to neuronal subtype identity. In another study from the same group, H3K9me3 was shown to be altered by repeated cocaine in the NAc (Maze et al., 2011). ChIp-seq analysis revealed that H3K9me3 was predominantly within intergenic regions and at repetitive genomic sequences. Similar finding was described following morphine treatment, with a specific decrease of G9a associated with diminished levels of H3K9me2 in the NAc mostly in intergenic regions and repetitive sequences (Sun et al., 2012). A ChIP-seq analysis was performed to further identify targets of G9a and, data confirmed previously described genes associated with morphine effects as well as novel targets, including glutamatergic signaling genes like Grin2a, Grip1, *G*rm5, and Grm8. Together, these findings suggest a critical role for histone methyltransferase, whose control of basal patterns of gene expression is altered by exposure to cocaine and morphine.

Interestingly, a recent study investigated the role of histone Arg (R) methylation in cocaine action and showed a decreased expression of protein-R-methyltransferase-6 (PRMT6) and its associated histone mark (H3R2me2a) in the NAc of mice or rats exposed to cocaine or in human addicts (Damez-Werno et al., 2016). This down-regulation is specifically observed in *Drd2* expressing neurons, and opposite in Drd1. Using ChIP-seq, they identified Src kinase signaling inhibitor 1 (Srcin1) as a target for reduced H3R2me2 binding, highlighting the effect of cocaine on another histone mark.

Altogether, histone acetylation and methylation are playing an important role in drug adaptations in the NAc, however, more studies are necessary to propose a molecular mechanism for such adaptations, particularly with distinct drugs of abuse.

5. Epigenetic transmission

Exposure to drugs of abuse during a sensitive period for brain development may be critical for epigenetic changes as they could be exacerbated later on in the adulthood or even become transgenerational (Bale, 2015). Such studies are highlighted in the tables (see * in Tables) and some of them discussed below.

5.1. Gestational exposure

Genome-wide approaches have revealed that in utero drug exposure could induce epigenetic modifications. Most of these focus on nicotine effects as numerous studies showed fetal brain development alterations due to maternal smoking (Banderali et al., 2015). Transcriptomic analysis was performed on offspring cortical samples from mice exposed to nicotine (200 μg/ml) from parental mating to weaning, followed by a three-month withdrawal (Jung et al., 2016). The authors identified *Ash21*, a gene involved in histone methylation, as the most up-regulated transcripts by nicotine exposure during development. They further examined genome-wide changes in H3K4me3 by ChIP-seq analysis and identified most alterations induced by developmental nicotine exposure at promoter regions of genes involved in glutamate neurotransmission. Interestingly, knockdown of Ash2l abolished nicotine-mediated alterations of dendritic complexity and decreased nicotine-dependent changes in passive avoidance behavior. These data highlighted Ash2l, forming a complex together with the transcription factor *Mef2c*, as critical targets for nicotine to alter neuron morphology and alter persistent behavior during development. Altogether, these genome-wide approaches point to a novel mechanism of gene regulation during brain development involving H3K4me3 epigenetic mark.

In a study examining the impact of e-cigarette smoke exposure during early life development (e-cigarette aerosol, 3 h/day; 5 day/week until postnatal day 4–6, with or without 13–16 mg/ml nicotine), the authors analyzed gene expression alterations using RNA-seq in the mouse offspring frontal cortex (Lauterstein et al., 2016). They described pathways with common gene networks related to cancer, organismal injury and abnormalities, and gastrointestinal disease. Interestingly, sex-dependent transcriptional changes were observed following aerosol exposure, with or without nicotine. In another study examining maternal nicotine exposure (nicotine in drinking water during gestation, 200 μg/ml), the authors examined transcriptional profiling (RNA-seq) on laser dissected *Pomc* neurons from the offspring (Silva et al., 2016). This approach revealed one consistent change, the upregulation of Gm15851, a lncRNA of yet unidentified function. In a global DNA methylation profiling (DNAm microarray) of human dorsolateral prefrontal cortex of nicotine exposed fetuses, the authors investigated the role of DNAm in adaptations to nicotine effects and gestational age (Chatterton et al., 2017). Two hypomethylated regions within *SDHAP3* (involved in mitochondrial membrane function) and *GNA15* (encodes a G protein subunit) promoters have been identified specifically in exposed -fetuses. Interestingly, the authors observed an increase of *SDHAP3* transcript only in exposed -fetuses between early and late development. A similar finding was noted for *GNA15*, with a larger increase in exposed versus unexposed -fetuses. These data suggest that maternal nicotine exposure induces both gene expression and DNA methylation modifications during cortical development (Chatterton et al., 2017). Interestingly, modification of the differentially methylated region (DMR) levels of these two genes has been also observed in autism and schizophrenia studies in cerebellum and cortical areas (Ladd-Acosta et al., 2014; Nardone et al., 2014; Wockner et al., 2015). Altogether, this highlight a link between DNA methylation and neurodevelopmental diseases, and such implication may also apply to SUD.

Prenatal ethanol effects have been examined in rats using RNA microarray (Middleton et al., 2012) or RNA-seq (Ignacio et al., 2014), and specific genes or miRNA have been identified in amygdala and NAc. Interestingly, these regulations involving signaling pathways could participate in the social motivation deficits seen in adolescent rats produced by prenatal ethanol, an effect that was reversed by a social enrichment in the offspring. Another study using genome-wide analysis of gene expression in the mouse hippocampus revealed altered expression of 23 genes and 3 miRNAs in a gestational ethanol-exposure model (Marjonen et al., 2015), including MiR138-2 also detected in the previous study, but with an opposite regulation. These distinct results may reflect differences in duration and timing of exposure. Interestingly, the authors identified a Histone cluster 1 H2ai, which was down regulated by ethanol exposure, indicative of altered-chromatin conformation by alcohol, across generation.

5.2. Transgenerational inheritance

Transgenerational inheritance, reflecting transmission to unexposed offspring, has also been recently explored in the addiction field. Initial studies investigated candidate genes following alcohol, morphine or cocaine exposure. For example, Pomc gene hypermethylation caused by fetal alcohol exposure was transmitted to offspring through male germline (Govorko et al., 2012). Analysis of the progeny of adolescent female rats exposed to morphine showed an increase of D2 and kappa opioid receptor expression in two generations of mice, suggesting epigenetic adaptations (Byrnes et al., 2013). In another study, reduced cocaine intake was observed specifically in male offspring of cocaine-experienced males, coupled with an increased association of acetylated histone H3 (H3K9K14ac2) with BDNF promoters (Vassoler et al., 2013). This epigenetic adaptation was associated with increased baseline anxiety in these animals, which was unaltered by subsequent cocaine exposure (White et al., 2016).

More recent studies explored epigenetic inheritance using genome-wide approaches to better investigate molecular targets or pathways modulated in progeny. In order to evaluate the impact of smoking marijuana on subsequent offspring, one study examined the effects of parental THC exposure (during rat adolescence) on DNA methylation in the NAc of adult F1 unexposed rats using a bisulfite-sequencing approach. The authors identified 406 hypermethylated and 621 hypomethylated DMRs across the genome. Interestingly, the majority of DMRs were detected in gene bodies and downstream of transcriptional start sites, particularly within genes involved in the glutamatergic synaptic regulation (Watson et al., 2015). The DMR-associated genes exhibited altered mRNA expression in the NAc. A gene network centered on *Dlg4* coding for PSD95, a scaffolding protein involved in synaptic plasticity was identified, together with genes involved in glutamatergic neurotransmission. *Dlg4* gene is regulated by epigenetic factors in aging and neurodegenerative diseases like Alzheimer's disease, Huntington's disease and schizophrenia (Bustos et al., 2017). Notably, PSD95 plays a major role in the formation of synapses during specific time points of neurodevelopment and regulates synaptic function that influences behavioral phenotypes in schizophrenia (Coley and Gao, 2018). Alteration of DNAm of *Dlg4* by parental THC exposure could therefore be associated with an increased risk of schizophrenia in cannabis consumers (Hudson et al., 2018). In a similar approach, other authors have analyzed the effect of parental methamphetamine exposure on hippocampal samples of offspring using DNAm microarray targeting annotated CpG Islands and promoter regions (Itzhak et al., 2015). 545 CpG islands and 156 promoter region hypermethylated DMRs induced by in utero methamphetamine exposure have been identified. Gene ontology analysis in the DMR highlighted "cerebral cortex GABAergic interneuron differentiation" for hypermethylated DMRs and "embryonic development" for hypomethylated ones. These results suggested that the observed phenotypes in F1 generation, including enhanced response to cocaine-conditioned reward and hyperlocomotion, and reduced fear conditioning, could be the result of abnormal brain development.

Another study investigated modification of DNAm profile (Bis-Seq) in hypothalamus of unexposed offspring of rats, whose parents were

exposed using a binge alcohol protocol at adolescence (Asimes et al., 2017). The authors showed altered epigenetic modifications in these naïve rats which varied depending on parental exposure. Indeed, differentially methylated cytosines were distinct between offspring, depending on which parent was exposed to ethanol. In addition, the highest number of hypermethylated and hypomethylated regions identified was observed when both parents were exposed to ethanol. Finally, less is known about morphine exposure on offspring and only one study described transcriptomic adaptations (RNA-seq) using maternal morphine exposure (Vassoler et al., 2017). The authors mainly observed altered gene expression in the NAc for genes specifically involved in synaptic plasticity and neural development associated with a higher resistance to self-administer morphine in the offspring.

In summary, all these studies highlight that prenatal exposure to drugs of abuse alters neural development and synaptic plasticity, as well as phenotypic behavioral responses. The transmission to the offspring seems to involve epigenetic modifications, but the mechanistic process of these regulations at the chromatin level still needs to be further addressed, including with comparison to transcriptomic profiling. Investigation of the persistence of these regulations by examining regulations in subsequent generations (F2 and F3) would be helpful for a more comprehensive interpretation of these mechanisms. Human studies have examined regulation in blood samples (Cecil et al., 2016a; Markunas et al., 2014) for alcohol or nicotine early exposure and highlighted epigenetic changes but direct correlations with modifications occurring in brain structures may be difficult to establish.

6. Developping tools for neuroepigenetic studies

6.1. Chromatin 3D structure and psychostimulant addiction

More recent studies have used sophisticated approaches to decipher the architecture of the chromatin in response to drugs of abuse. One study raised the question of the alteration of 3D architecture of chromatin induced by cocaine treatment, at genes showing epigenetic changes (Engmann et al., 2017). Using FAC sorting and viral mediated approaches, the authors found that *Auts2*, a gene linked to cognitive disorders, was enriched in *Drd2* medium spiny neurons in mice. An increased expression was also detected in cocaine-addict post-mortem NAc samples. In mouse NAc, using chromosome conformation capture-on-chip (4C-seq) investigating one specific locus vs all loci, the authors found that chromatin looping connected *Auts2* predominantly with a brain-specific calcium binding gene (*Caln1*) in basal conditions, an interaction that was disrupted following chronic cocaine treatment. Using bisulfite sequencing, they showed increased DNAm at the vicinity of *Auts2* interaction site. They further manipulated DNAm using CRISPR-genome editing in Neuro2a cells, targeting DNMT activity at the interaction site between *Auts2* and *Caln1*. This brought the evidence that DNAm alteration on *Auts2* gene could modify gene expression of a distant chromatin interaction partner, *Caln1* (Engmann et al., 2017). Even if this was shown in vitro due to current technical limitation in vivo, it demonstrated a causality between interaction and expression. Future technical improvement will certainly allow testing this mechanism directly in brain structures. These new findings provide novel insight for explaining the impact of epigenetic modifications on gene expression. Modification of expression in response to drugs may involve alteration of the 3D structure of chromatin at gene regulatory regions.

6.2. Epigenetic-based therapeutic interventions

Epigenetic modifications have been proposed as potential biomarkers for SUD (Cecil et al., 2016b). DNAm patterns in human blood cells may be used to identify vulnerability towards addiction in individuals. Hypermethylation in the 3′-protein-phosphatase-1 G gene locus has been associated with alcohol use disorder (Ruggeri et al., 2015). But whether these modifications are specific to blood cells or to a particular drug is still not clear.

Epigenetic regulations may explain the maintenance of long-term adaptation and a better understanding of the mechanisms across drugs of abuse will help highlight targets for relapse treatment. Manipulation of histone acetylation levels using pharmacological inhibitors of HDACs was assessed in preclinical cocaine studies. Such HDAC inhibitors reduced cocaine intake and motivation in self-administering rats while no effect of the inhibitor was observed on sucrose intake (Romieu et al., 2008). Reduced reinstatement of cocaine-seeking behavior was also observed following 3 weeks of abstinence (Romieu et al., 2011). Extinction of cocaine-induced CPP was accelerated following a similar treatment (Malvaez et al., 2010). In contrast, opposite results were obtained in other studies on cocaine intake (Sun et al., 2008) and cocaine-induced hyperlocomotion (Kumar et al., 2005). These results point to the importance of the specificity of these inhibitors or the type of treatment used, that may explain distinct effects on addictive responses. Therefore, the need for selective inhibitors of HDAC and controlled paradigms seem crucial to specifically target subtypes of HDAC in therapeutic approaches. Also, differential regulations were observed, depending on the targeted brain structure or the type of drug, as illustrated for HDAC5 with distinct regulations following methamphetamine in DS compared to cocaine in NAc (see above (Li et al., 2017). These observations reveal the complexity of such therapeutic strategies. Another approach could be to target histone acetyltransferase. These enzymes, like CBP, play a role in cocaine action in the NAc (Malvaez et al., 2011) but no study has evaluated their potential efficacy in drug-induced responses. Activation of CBP has recently been shown to reverse deficits in memory processes and promote neurogenesis (Chatterjee et al., 2013). Effects of activation of histone acetyltransferase on drug-induced phenotypes could be proposed as a therapeutic approach.

Several tools are currently available to edit epigenetic factors, like ZFP, TALENs and CRISPR (reviewed in (Waryah et al., 2018). Such approaches provide accurate modification of a given type of epigenetic marks at a precise gene locus, allowing subtle changes to be detected. In vivo, such tools are able to reverse memory deficits in a mouse model of Alzheimer's disease (Bustos et al., 2017). In addictive disorders, one recent study by Nestler's group examined the role of cocaine-induced histone modifications at the gene locus for cyclin-dependent kinase 5 *cdk5*, a critical gene in reward related behaviors (Heller et al., 2016). The authors used zinc finger proteins-genome editing targeting H3K9/14ac, an active mark or H3K9me2, a repressive mark, in vivo and demonstrated a causal role of *Cdk5* epigenetic remodeling in NAc in *Cdk5* gene expression as well as in reward and stress behaviors. Their results revealed novel indicators of a role of this gene in addictive behaviors and, highlighted that targeted epigenetic remodeling approaches represent useful tools for studying molecular changes involved in addictive responses. Neuroepigenetic editing thus represents an emerging tool that will be developed to further investigate cell-type and gene specific adaptations.

7. Concluding remarks

In summary, genome-wide studies have highlighted the implication of several types of neuroepigenetic modifications associated with drugs of abuse, which may have an impact on addictive behaviors. Histone acetylation and methylation are clearly altered by psychostimulants. DNAm and hydroxymethylation represent dynamic mechanisms that may affect enhancers, promoters, splice-sites with different outcome on gene expression. Further, analysis of 3D structure of chromatin brings new insight into the mechanism by which drugs alter gene expression. Combining chromatin architecture analyses with epigenetic and transcriptomic analyses is crucial for a better understanding of these adaptations.

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II. Annex 2

Analysis of S18297 RNA sequencing data : second experiment

Celine Keime

1 Data analysed in this report

Figure 1 on the following page represents the total number of sequenced reads in each sample (50 bp reads).

Table 1 lists all samples analysed in this report, together with their associated experimental conditions.

Table 1: **Samples analysed in this report and their experimental conditions.**

Sample ID	Sample name	Condition
KTBT9	12F_1	Food
KTBT10	12F_2	Food
KTBT11	12F_3	Food
KTBT12	12F_4	Food
KTBT13	12SF_1	Sucrose12
KTBT14	12SF_2	Sucrose12
KTBT15	12SF_3	Sucrose12
KTBT16	12SF_4	Sucrose12
KTBT17	24SF_1	Sucrose24
KTBT18	24SF_2	Sucrose24
KTBT19	24SF_3	Sucrose24
KTBT20	24SF_4	Sucrose24

2 Preprocessing

Reads were preprocessed in order to remove adapter, polyA and low-quality sequences (Phred quality score below 20). After this preprocessing, reads shorter than 40 bases were discarded for further analysis. These preprocessing steps were performed using cutadapt [1] version 1.10. Reads were mapped to rRNA and spike sequences using bowtie [2] version 2.2.8, and reads mapping to rRNA or spike sequences were removed for further analysis.

Figure 2 on page 3 provides the proportion of remaining reads after each preprocessing step.

Figure 3 on page 4 provides the number of different[1] and unique[1] reads in each sample after the preprocessing step.

3 Mapping

Reads were mapped onto the rn6 assembly of *Rattus norvegicus* genome using STAR [3] version 2.5.3a. Figure 4 on page 5 provides a summary of mapping results.

Figure 5 on page 6 represents read coverage over genes in all samples (coverage was computed for each gene percentile using geneBodyCoverage from RSeQC [4] version 2.6.4).

[1]For a given sample, the set of unique reads contains reads found only once in this sample and the set of different reads contains all distinct reads, whatever their occurrence number. For instance, for the following set of reads $\{A, B, C, C, D, E, F, F, F, G\}$, the set of unique reads is $\{A, B, D, E, G\}$ and the set of different reads is $\{A, B, C, D, E, F, G\}$.

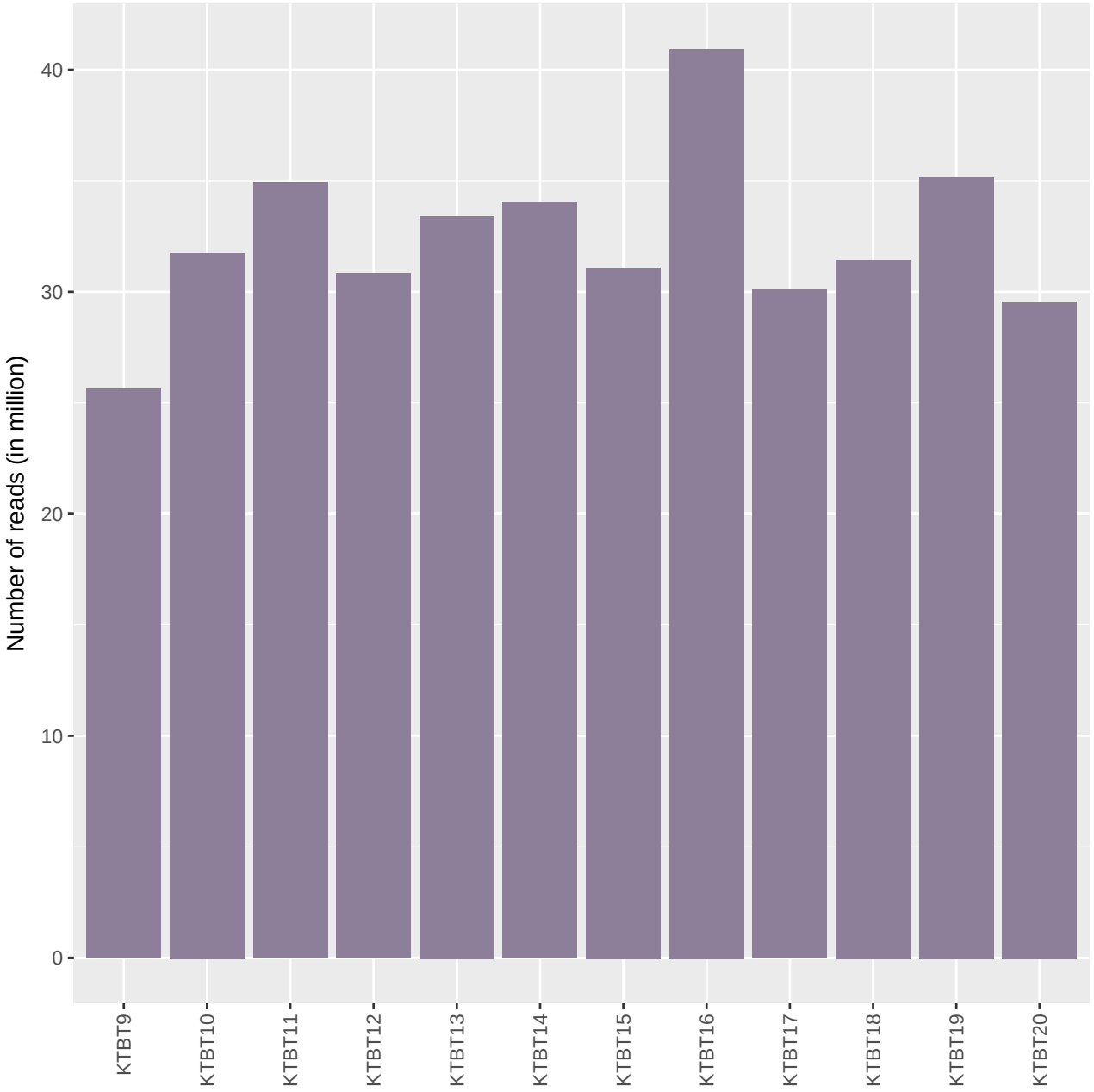

Figure 1: **Number of sequenced reads in each sample.** This barplot represents the total number of sequenced reads (in million, y-axis), in all samples (x-axis).

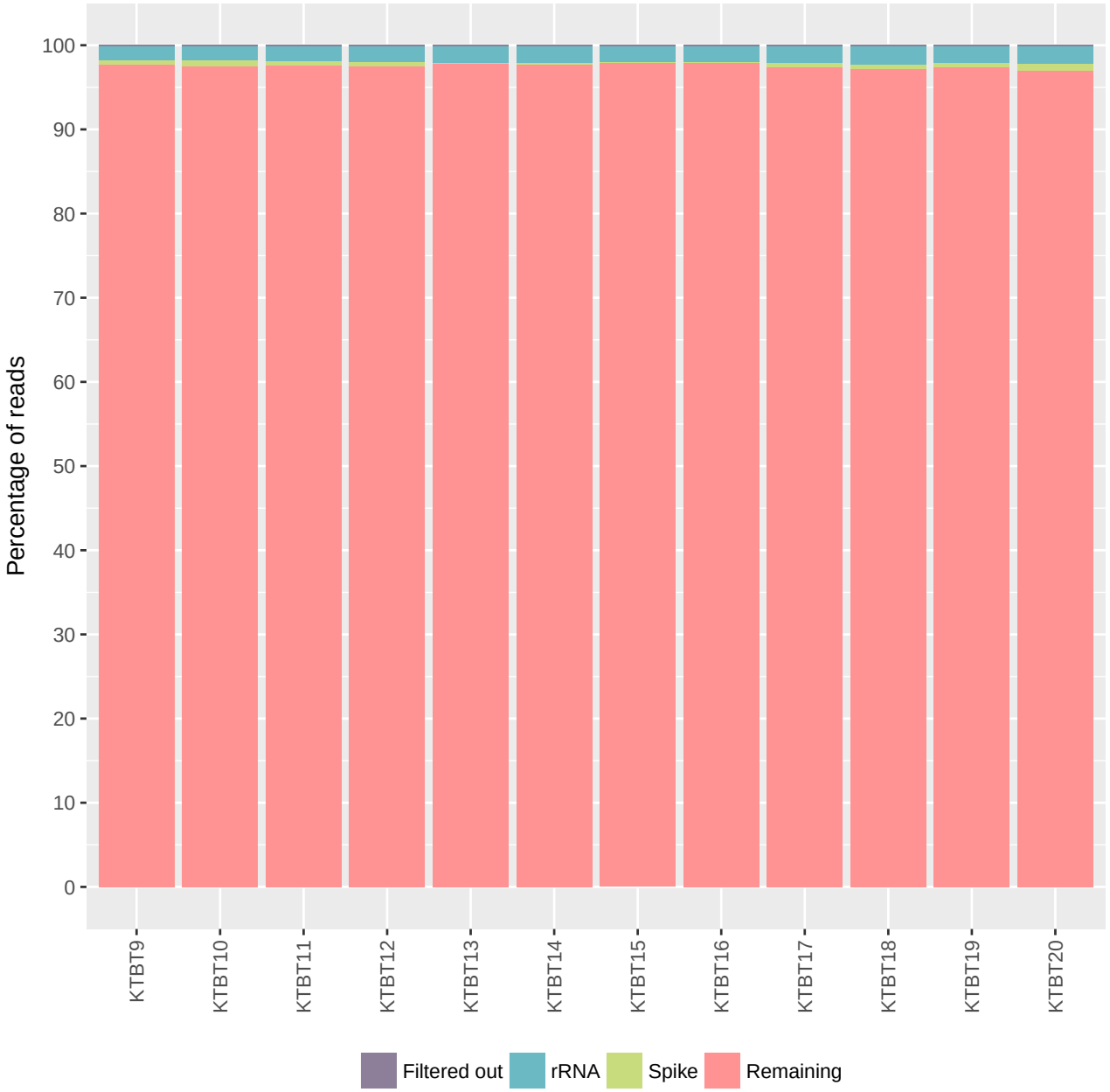

Figure 2: **Summary of preprocessing results.** "Filtered out" represents the percentage of reads shorter than 40 bases discarded after adapter and low-quality sequences (Phred quality score below 20) removal. "rRNA" represents the percentage of reads mapping to rRNA sequences. "Spike" represents the percentage of reads mapping to spike-in sequences. "Remaining" indicates the percentage of reads that remain after all preprocessing steps. All percentages were calculated relative to the total number of sequenced reads in each sample.

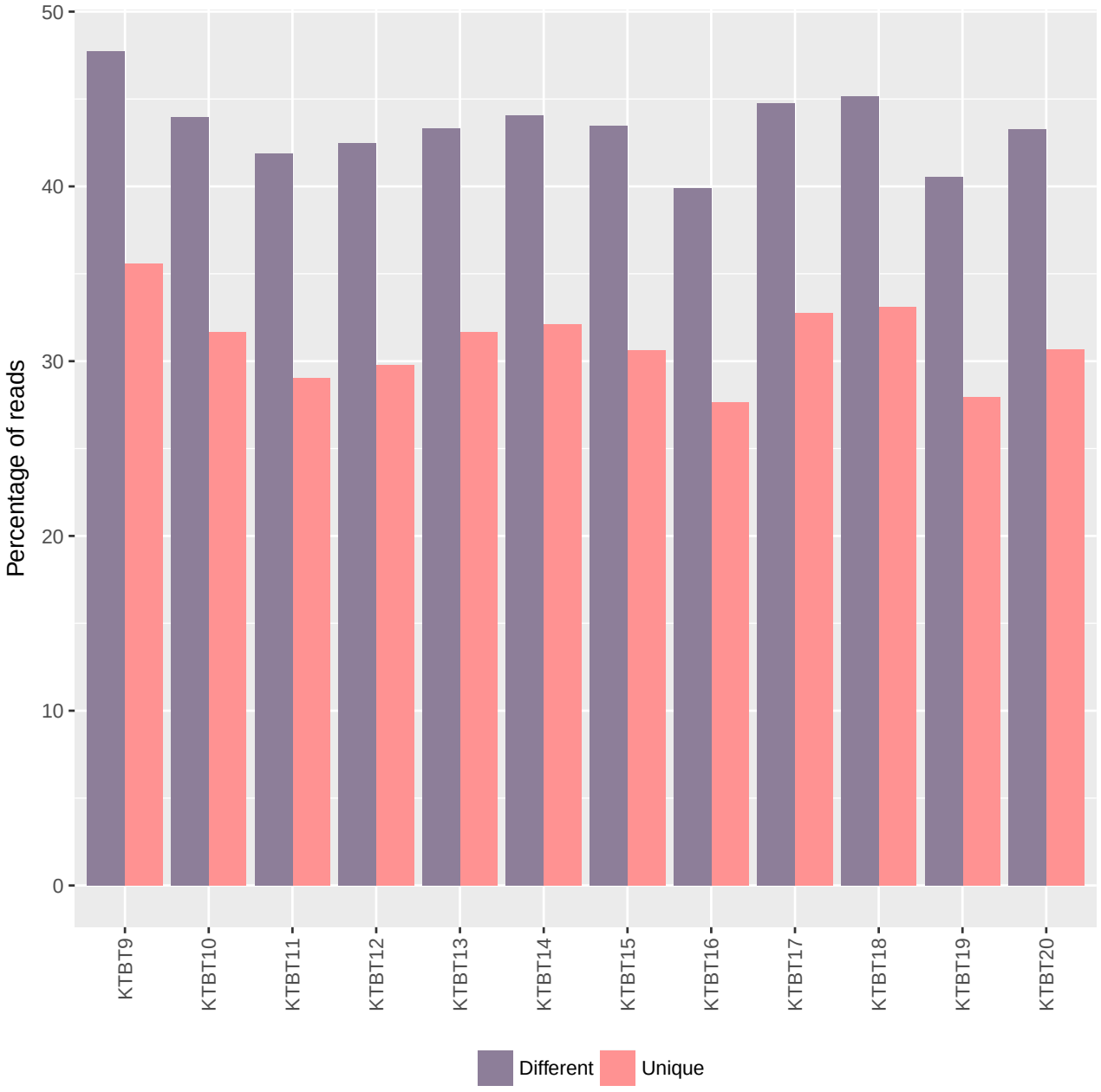

Figure 3: **Percentage of different and unique reads in each sample.** These percentages were calculated relative to the number of reads after preprocessing step.

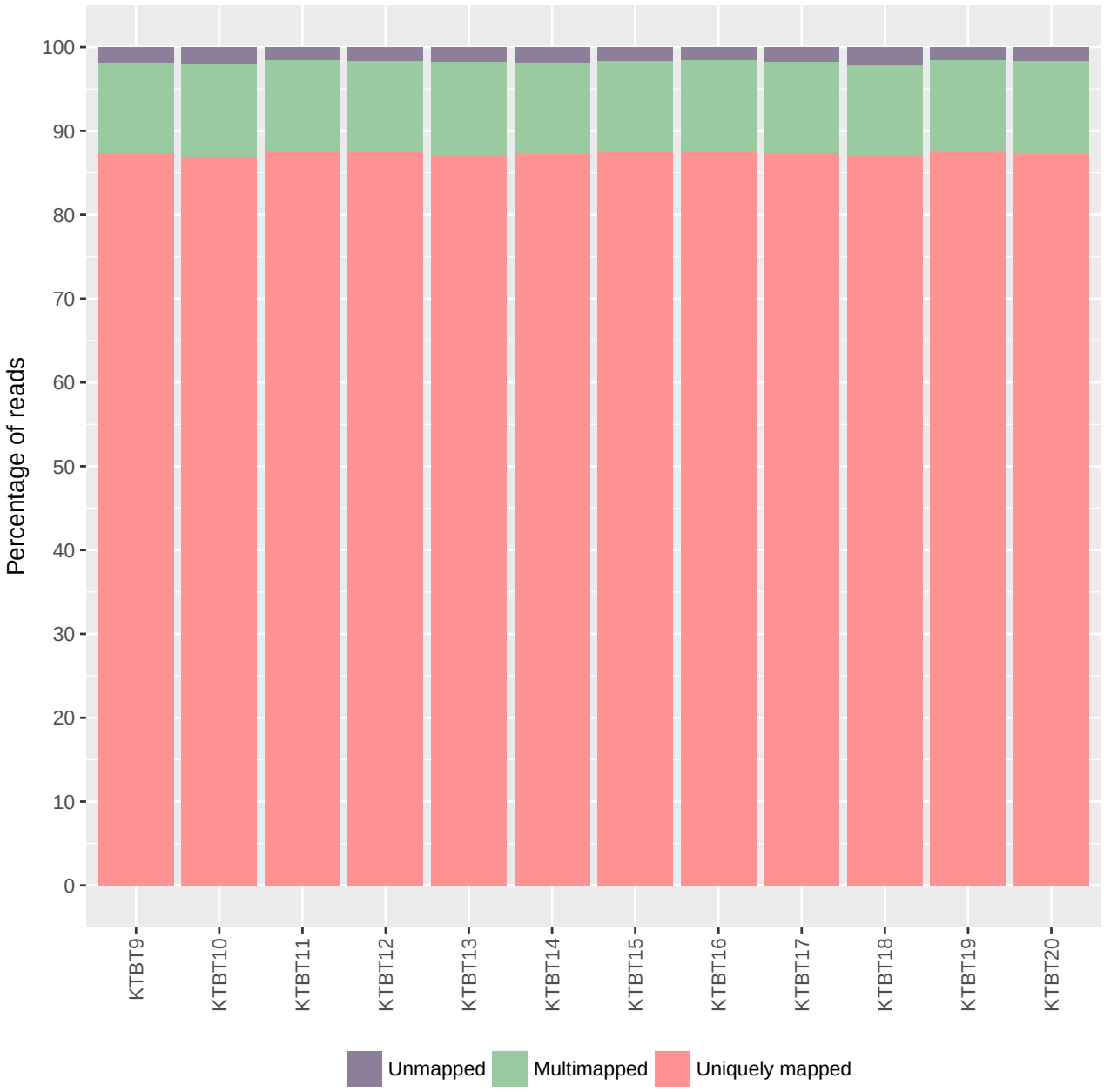

Figure 4: **Summary of mapping results.** This barplot represents the percentage of reads mapped only once on the genome (uniquely mapped), mapped at several locations on the genome (multi-mapped), or not mapped onto the genome (unmapped). These percentages were calculated relative to the number of input reads (i.e. reads kept after preprocessing).

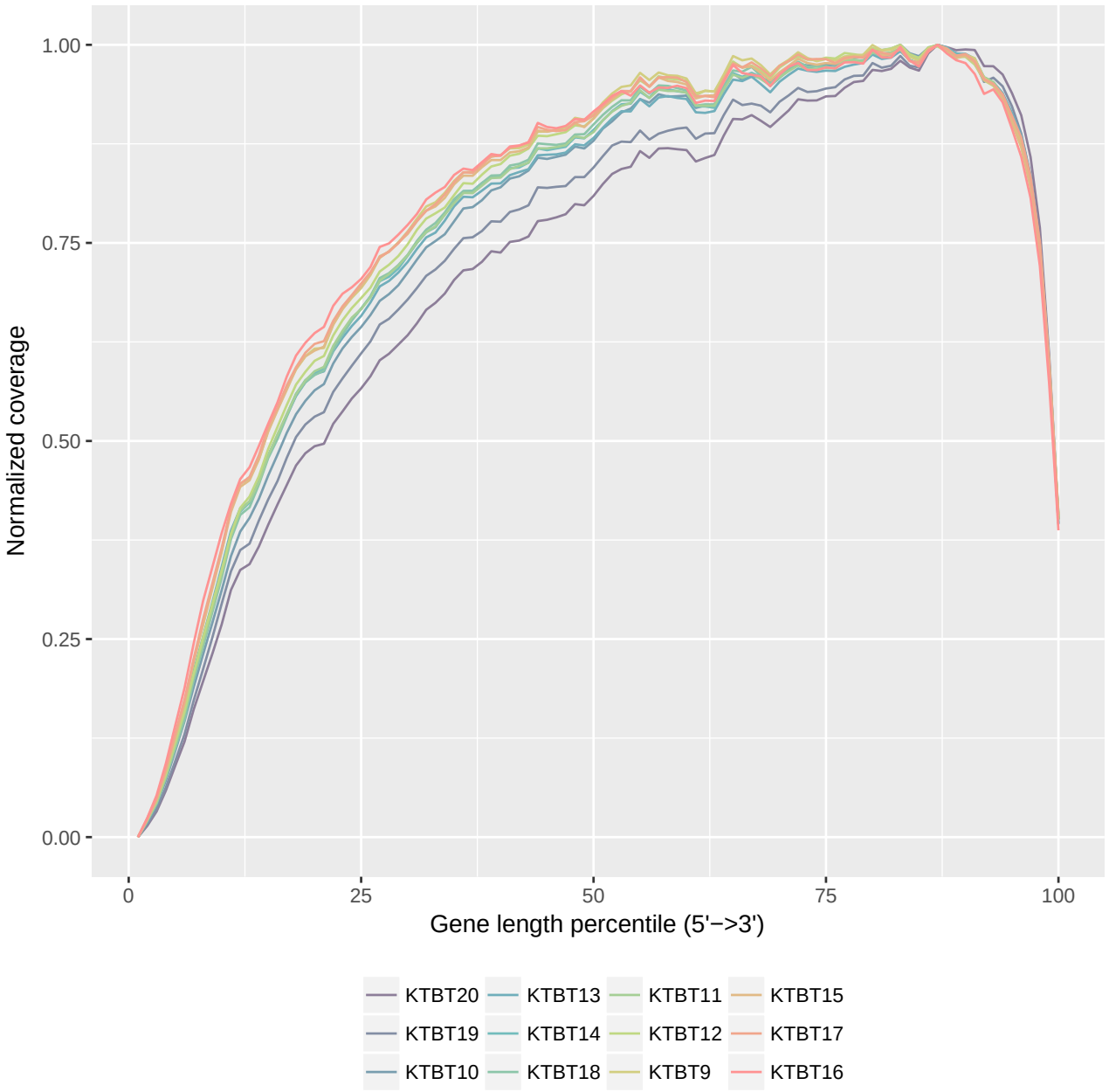

Figure 5: **Read coverage over genes in all samples.** This plot represents the normalized coverage (y-axis) at all percentiles of gene length (x-axis). Genes with mRNA length below 100bp were skipped from this analysis. In the legend, samples are ordered according to their Pearson's skewness coefficient (samples with more skewness are displayed at the begining of the legend).

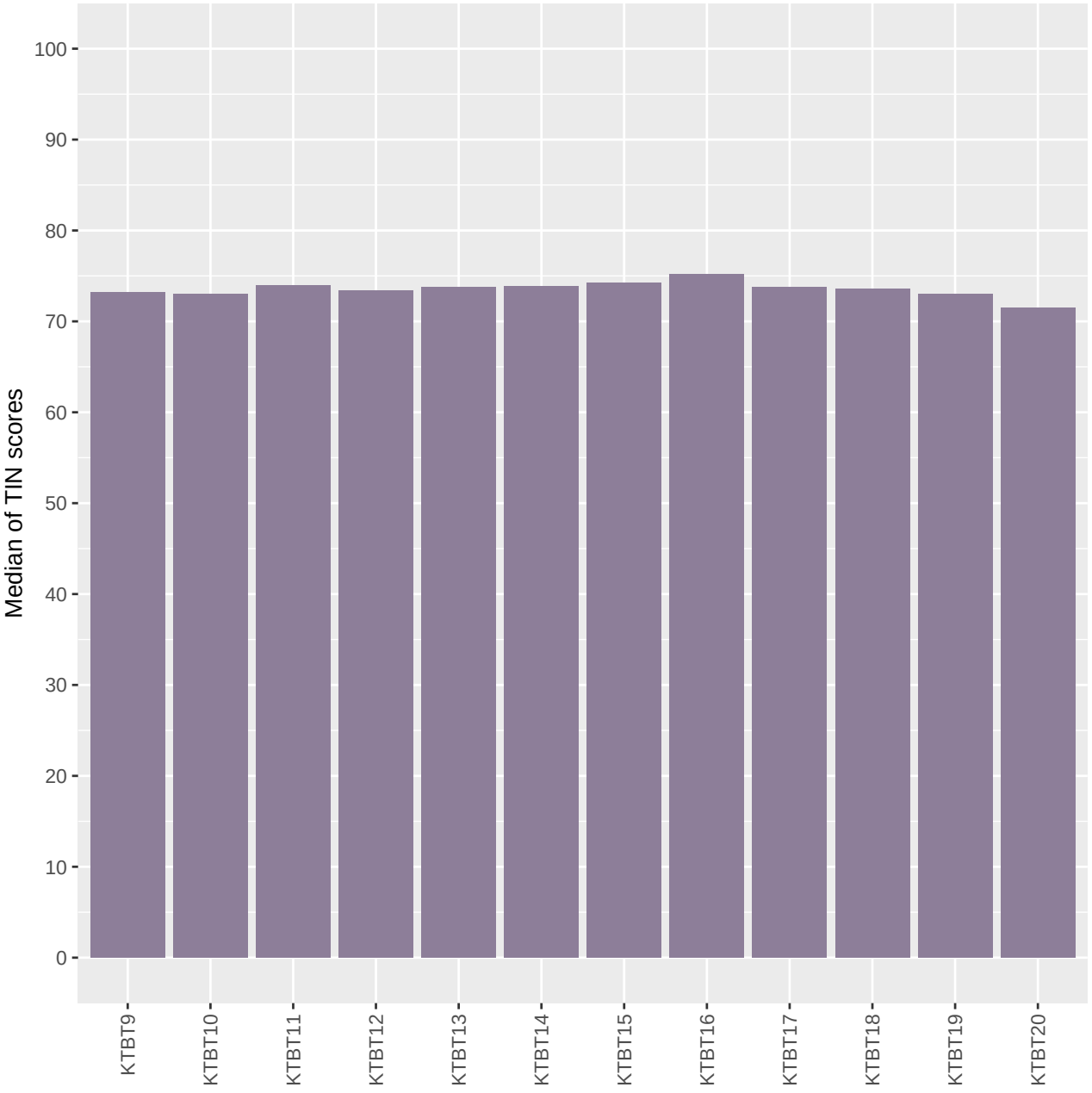

Figure 6: **Median of TIN scores for each sample.** This barplot represents the median of TIN scores (y-axis), in all samples (x-axis). TIN score is the percentage of transcript that has uniform read coverage and ranges from 0 (the worst) to 100 (the best). Transcript with a number of mapped reads below 10 were skipped from the analysis.

Figure 6 on the preceding page provides the median of transcript integrity numbers (TIN) [5] computed across all transcripts with at least 10 mapped reads. TIN is a metric dedicated to capture the uniformity of coverage for a given transcript, and thus is a measure of RNA integrity. TIN score is the percentage of transcript that has uniform read coverage and ranges from 0 (the worst) to 100 (the best).

Figure 7 on the next page provides the proportion of uniquely aligned reads across exonic, intronic and intergenic genomic regions (using annotations from Ensembl release 95). When genome features overlapped (e.g. the same region can be annotated as both exonic and intronic when different transcripts are overlapping), they are prioritized as: exonic > intronic > intergenic regions. For example, if a read was mapped to both exonic and intronic regions, it will be assigned to exonic region.

4 Quantification

Gene expression quantification was performed from uniquely aligned reads using htseq-count [6] version 0.6.1p1, with annotations from Ensembl version 95 and "union" mode[2]. Figure 8 on page 10 provides a summary of quantification results. Only non-ambiguously assigned reads have been retained for further analyses.

5 Data exploration

Figure 9 on page 11 provides an heatmap of sample-to-sample distances. The Simple Error Ratio Estimate (SERE) [7] coefficient that quantifies global RNA-seq sample differences has been used. A SERE coefficient of 0 indicates data duplication, a score of 1 corresponds to faithful replication (samples differ exactly as would be expected due to Poisson variation). If RNA-Seq samples are truly different, this coefficient is greater than 1 (overdispersion), and the more the coefficient is high, the more the samples are different.

Figure 10 on page 12 represents represents the first principal components of a Principal Component Analysis, showing the main sources of variance in the data.

6 Differential gene expression analysis

Comparisons of interest (listed in Table 2) were performed using the test for differential expression indicated in Table 2, proposed by Love et al. [8] and implemented in the Bioconductor package DESeq2 version 1.16.1.

Table 2: **Differential expression comparisons performed.**

Name of the comparison	Statistical test	Variable(s) taken into account in the model	Levels compared	Sample(s) filtered out
Sucrose12 vs Food	Wald	Condition	Sucrose12 vs Food	None
Sucrose24 vs Food	Wald	Condition	Sucrose24 vs Food	None
Sucrose12 vs Sucrose24	Wald	Condition	Sucrose12 vs Sucrose24	None

Genes with no p-value in the resulting file correspond to genes with high Cook's distance that were filtered out. Cook's distance is a measure of how much a single sample is influencing the fitted coefficients for a gene, and a large value of Cook's distance is intended to indicate an outlier count.

P-values were adjusted for multiple testing using the Benjamini and Hochberg method [9].

Genes with no adjusted p-value in the resulting file correspond to genes filtered out in the independent filtering step. Independent filtering based on the mean of normalized counts was performed in order to filter out those genes that have no or little chance of showing significance evidence of differential expression (without looking at their statistic). Indeed, genes with very low counts in all samples are not likely to be significantly differentially expressed. This independent filtering results in increased detection power.

Table 3 on page 16 provides the number of significantly differentially expressed genes in all comparisons.

Figures 11 on page 13 to 13 on page 15 represent the results of these comparisons.

[2] http://htseq.readthedocs.io/en/master/count.html

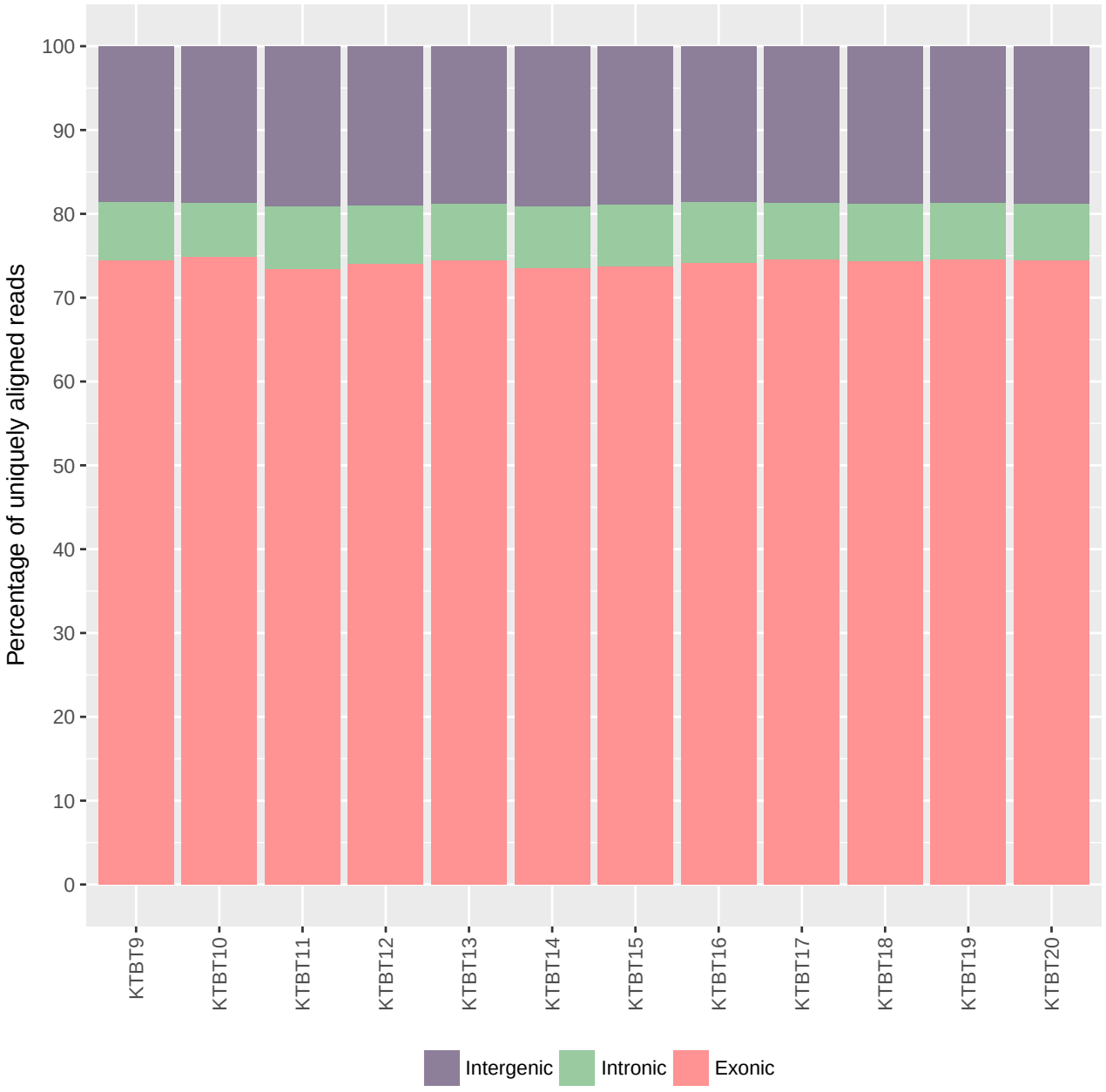

Figure 7: **Reads distribution over annotated genome features.** This barplot represents the proportion of reads aligned to exonic, intronic or intergenic regions, among all uniquely aligned reads.

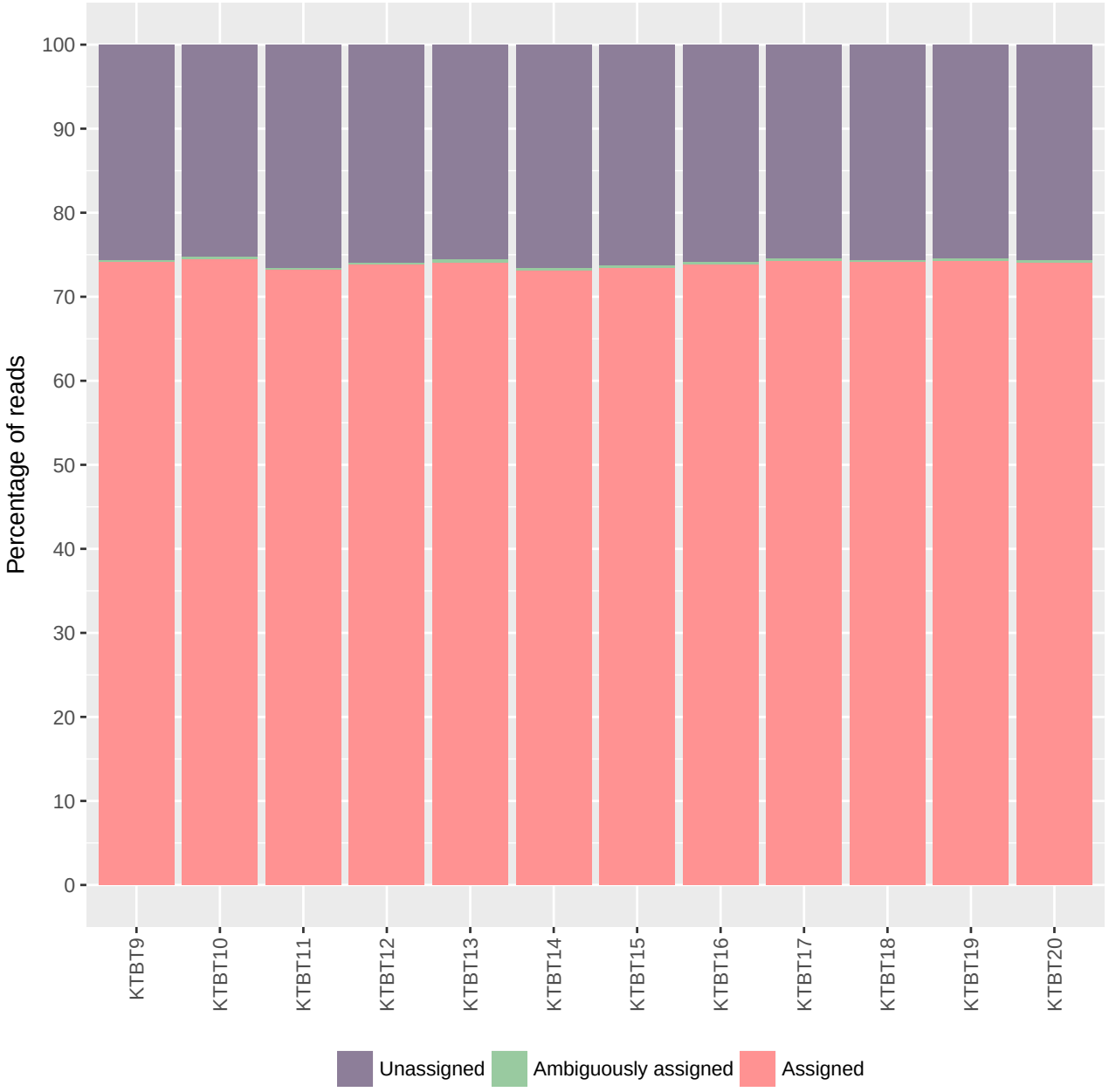

Figure 8: **Summary of quantification results.** This barplot represents the proportion of reads aligned to a genomic region corresponding to one annotated gene (Assigned), to more than one annotated gene (Ambiguously assigned) or to no annotated gene (Unassigned), among all uniquely aligned reads.

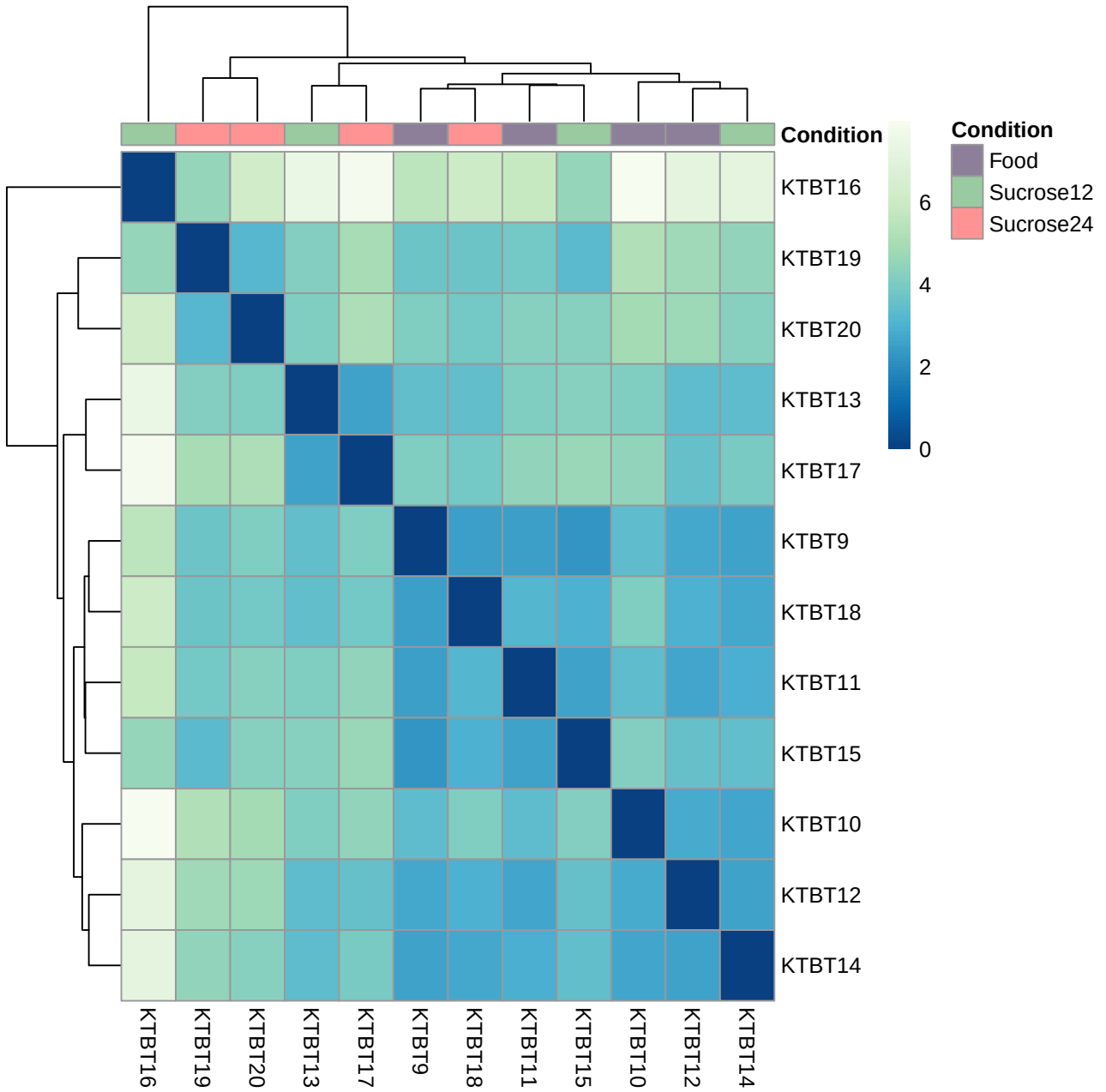

Figure 9: **Heatmap of sample-to-sample distances.** Sample-to-sample distances correspond to SERE [7] coefficient. Hierarchical clustering was performed using the Unweighted Pair Group Method with Arithmetic mean (UPGMA) algorithm.

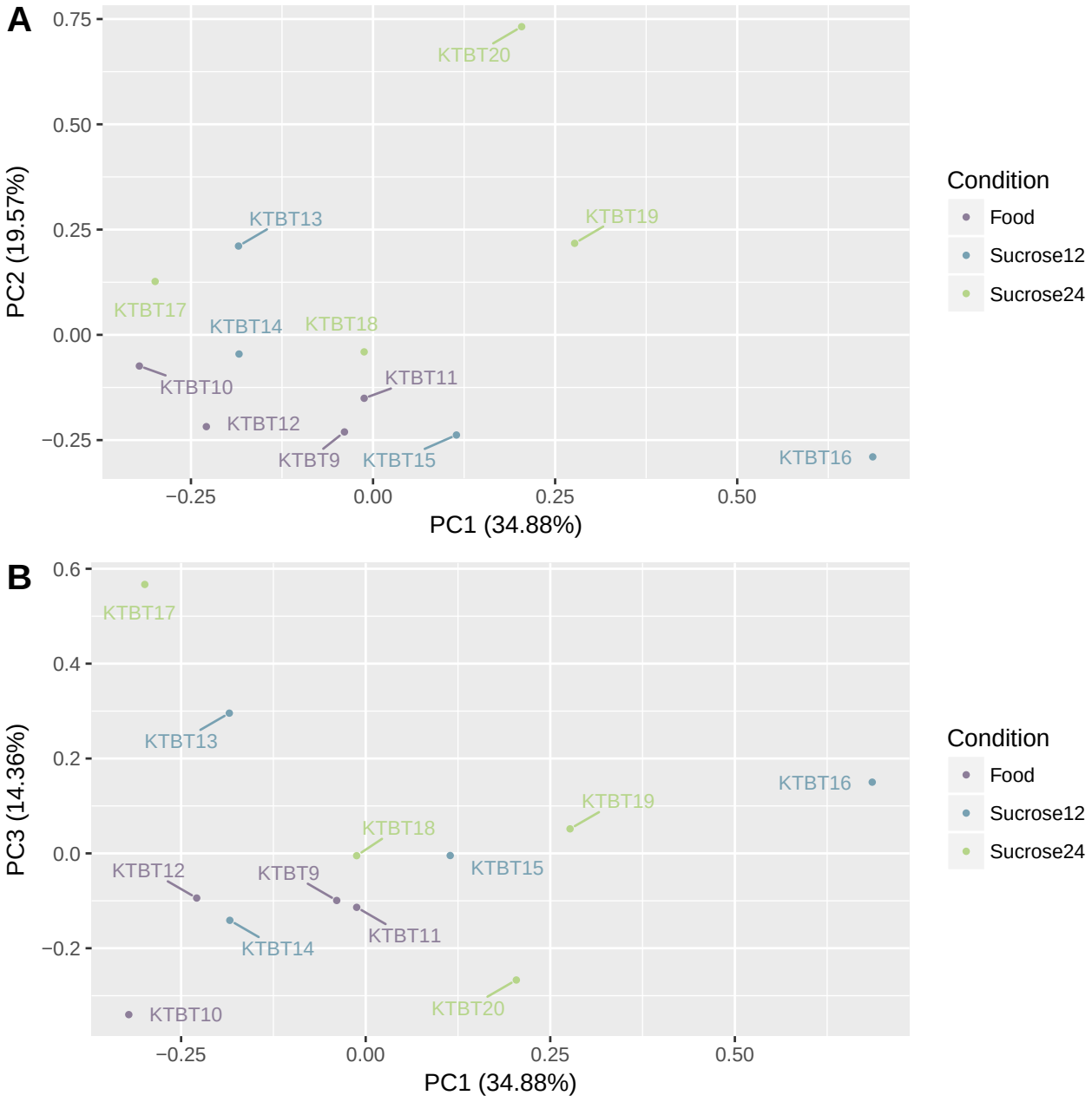

Figure 10: **Principal component analysis.** PCi axis represents the principal component i and the number into brackets indicates the percentage of explained variance associated with this axis. Principal Component Analysis was computed on regularized logarithm transformed data calculated with the method proposed in [8].

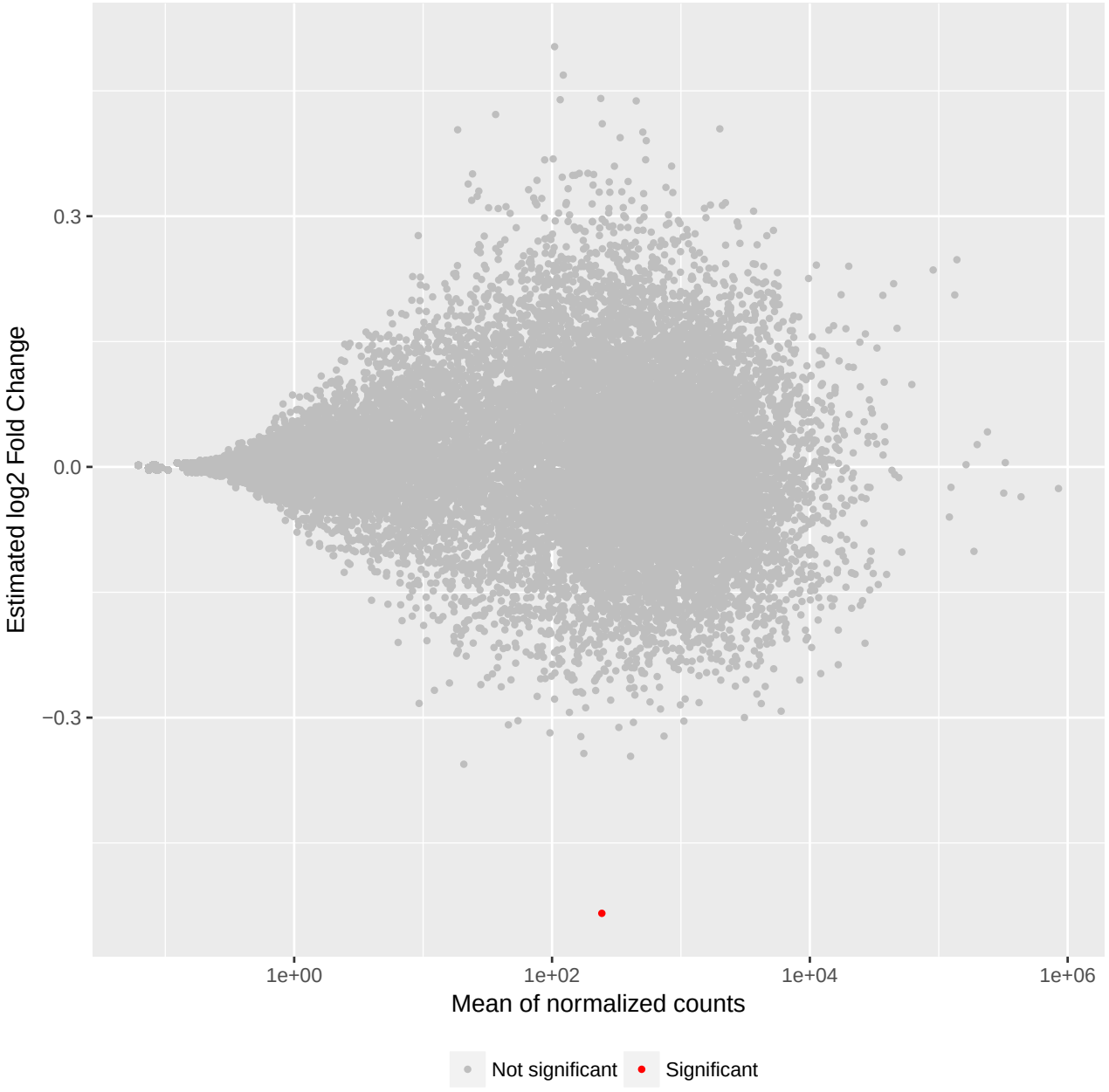

Figure 11: **Sucrose12 vs Food comparison.** MA-plot representing the estimated log2 Fold-Change as a function of the mean of normalized counts. Significant genes were selected using the following thresholds: adjusted p-value lower than 0.1.

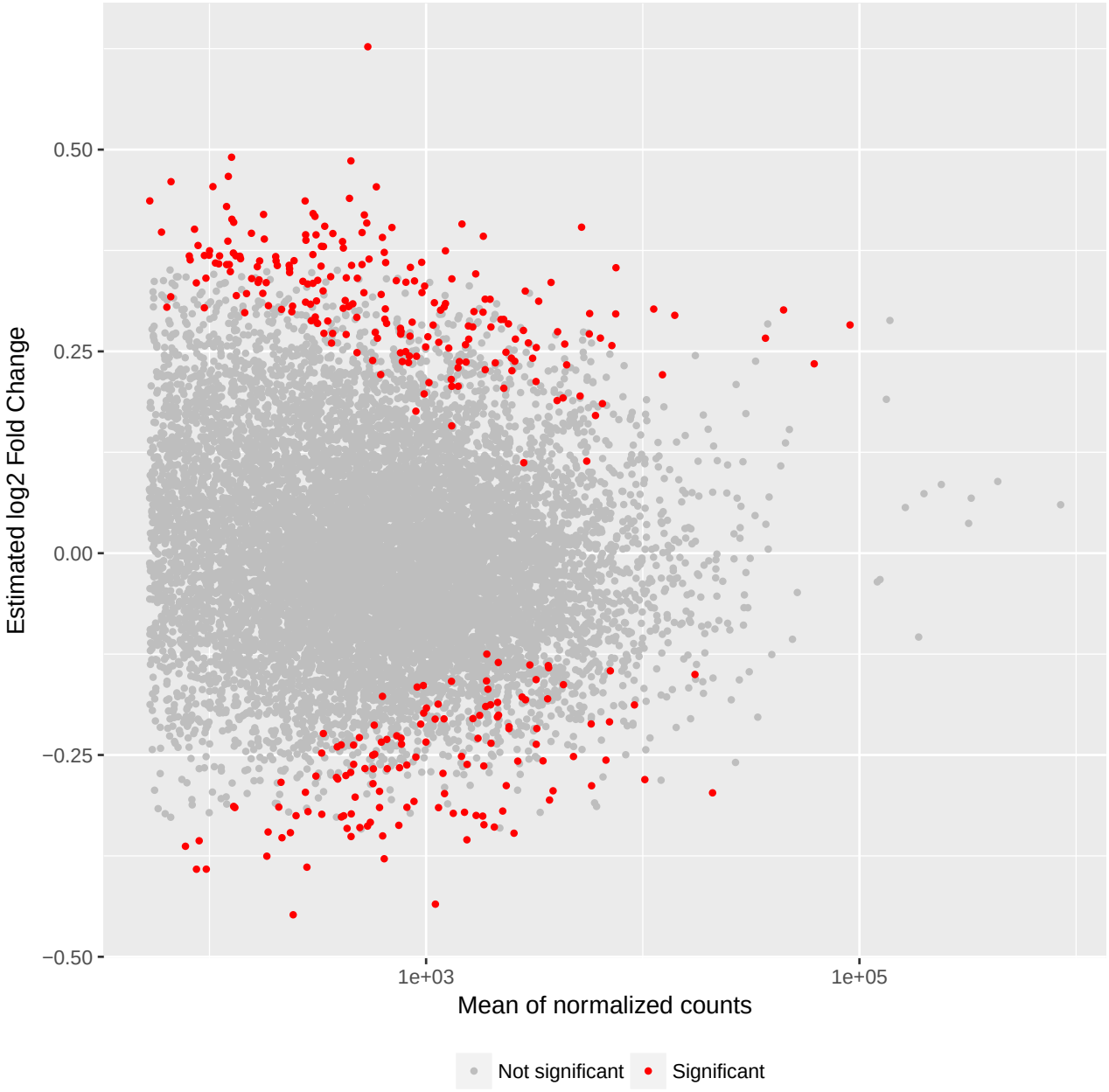

Figure 12: **Sucrose24 vs Food comparison.** MA-plot representing the estimated log2 Fold-Change as a function of the mean of normalized counts. Significant genes were selected using the following thresholds: adjusted p-value lower than 0.1.

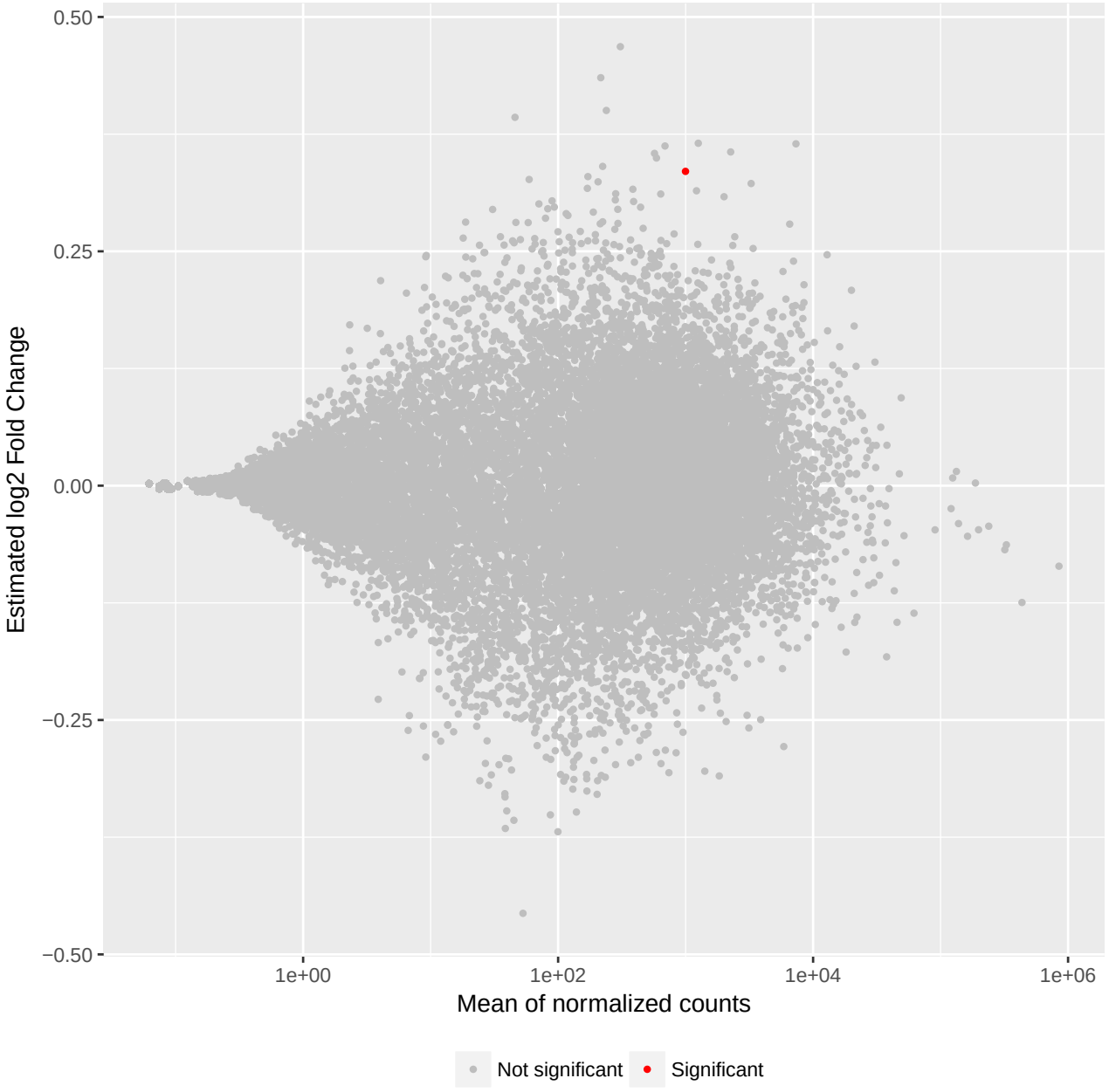

Figure 13: **Sucrose12 vs Sucrose24 comparison.** MA-plot representing the estimated log2 Fold-Change as a function of the mean of normalized counts. Significant genes were selected using the following thresholds: adjusted p-value lower than 0.1.

Table 3: **Number of significantly differentially expressed genes.** These genes were selected using the following thresholds: adjusted p-value lower than 0.1.

Name of the comparison (A vs B)	Number of over-expressed genes (A>B)	Number of under-expressed genes (A<B)	Number of significantly differentially expressed genes
Sucrose12 vs Food	0	1	1
Sucrose24 vs Food	214	128	342
Sucrose12 vs Sucrose24	1	0	1

7 Files delivered

7.1 Alignment files

For each sample, an alignment file in BAM format and the corresponding index (BAI format) are available. The BAM files can be opened using a genome browser, for example Integrative Genomics Viewer [3].

7.2 Result file

A TSV (tab-separated values) file provides raw read counts and normalized read counts for each gene together with gene annotations and the p-value, adjusted p-value and log2 fold-change for each performed comparison. This file contains only genes with at least one read count in one sample. It can be opened with a spreadsheet software like Excel or Calc. The "," character is used as decimal separator in numeric columns. This file contains the following columns:

Ensembl Gene ID Ensembl identifier of the gene, corresponding to Ensembl release 95.

Raw read counts Number of reads that have been assigned to the gene.

Normalized read counts Number of reads that have been assigned to the gene, normalized to make these counts comparable between samples, using the method described in [10].

Normalized read counts divided by median of transcripts length in kb Number of reads that have been assigned to the gene, normalized between samples and divided by transcript length in kb (calculated as the median of the length of all transcripts corresponding to this gene). These expression estimates can be compared across genes and samples.

Median of transcripts length Median of the length of all transcripts corresponding to this gene (in bp).

Gene name Common gene name.

Description Description of the gene.

Chromosome name Name of the chromosome where the gene is located.

Start gene position Start coordinate of the gene.

End gene position End coordinate of the gene.

Gene biotype Biotype of the gene as defined in Ensembl[4].

GO:biological process Biological process Gene Ontology terms associated with this gene. A biological process term describes a series of events accomplished by one or more organized assemblies of molecular functions.

GO:molecular function Molecular function Gene Ontology terms associated with this gene. A molecular function term describes activities that occur at the molecular level.

[3] IGV is freely available on `http://software.broadinstitute.org/software/igv`

[4] `https://www.ensembl.org/Help/Faq?id=468`

GO:cellular component Cellular component Gene Ontology terms associated with this gene. A cellular component term describes a location, relative to cellular compartments and structures, occupied by a macromolecular machine when it carries out a molecular function.

log2 FC Log2 of the expression fold change estimated, reflecting differential expression between the two compared conditions.

p-value P-value of the statistical test.

Adjusted p-value P-value of the statistical test, adjusted for multiple testing.

8 Version information

8.1 Version of used tools

Table 4 provides the tools used in GenomEast RNA-seq pipeline version 1.2.2 (used to perform the analyses described in the report) and their corresponding version.

Table 4: **Tools used for the analyses presented in this report.**

Tool	Release	Description
bowtie2	2.2.8	To align reads onto a set of reference sequences.
cutadapt	1.10	To trim low quality bases and adapter sequences from the reads and to remove too-short reads after trimming.
FastQC	0.11.5	To perform quality controls on the reads.
HTSeq	0.6.1p1	To compute the number of reads in annotated transcribed regions.
R	3.3.2	To perform statistical analysis, graphics and to generate this report.
RSeqQC	2.6.4	To perform quality controls on the alignments.
samtools	1.3.1	To manipulate SAM/BAM files.
STAR	2.5.3a	To perform spliced alignment of reads onto a reference genome.

8.2 Version of used R packages

```
## R version 3.3.2 (2016-10-31)
## Platform: x86_64-pc-linux-gnu (64-bit)
## Running under: Scientific Linux release 6.7 (Carbon)
##
## locale:
##  [1] LC_CTYPE=fr_FR.UTF-8       LC_NUMERIC=C
##  [3] LC_TIME=fr_FR.UTF-8        LC_COLLATE=fr_FR.UTF-8
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##
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##  [8] utils     datasets  base
##
## other attached packages:
##  [1] DESeq2_1.16.1              SummarizedExperiment_1.4.0
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```
##  [3] Biobase_2.34.0              GenomicRanges_1.26.4
##  [5] GenomeInfoDb_1.10.3         IRanges_2.8.2
##  [7] S4Vectors_0.12.2            BiocGenerics_0.22.0
##  [9] ggrepel_0.6.5               ggfortify_0.4.1
## [11] pheatmap_1.0.8              reshape2_1.4.2
## [13] VennDiagram_1.6.18          futile.logger_1.4.1
## [15] knitr_1.12.3                xtable_1.8-2
## [17] cowplot_0.8.0               ggplot2_2.2.1
##
## loaded via a namespace (and not attached):
##  [1] locfit_1.5-9.1       Rcpp_0.12.13         lattice_0.20-34
##  [4] tidyr_0.6.1          assertthat_0.1       digest_0.6.12
##  [7] R6_2.2.0             plyr_1.8.4           futile.options_1.0.0
## [10] backports_1.0.5      acepack_1.4.1        RSQLite_1.1-2
## [13] evaluate_0.10        zlibbioc_1.20.0      lazyeval_0.2.0
## [16] annotate_1.50.0      data.table_1.10.4    rpart_4.1-10
## [19] Matrix_1.2-7.1       checkmate_1.8.2      labeling_0.3
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## [28] RCurl_1.95-4.8       munsell_0.4.3        base64enc_0.1-3
## [31] htmltools_0.3        nnet_7.3-12          tibble_1.2
## [34] gridExtra_2.2.1      htmlTable_1.9        Hmisc_4.0-3
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## [40] gtable_0.2.0         DBI_0.6-1            magrittr_1.5
## [43] formatR_1.4          scales_0.4.1         stringi_1.1.2
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## [49] Formula_1.2-1        lambda.r_1.1.7       RColorBrewer_1.1-2
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## [55] colorspace_1.3-2     cluster_2.0.6        memoise_1.1.0
```

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UNIVERSITE DE STRASBOURG

RESUME DE LA THESE DE DOCTORAT

Discipline : **Neurosciences**

Présentée par : **De Sa Nogueira David**

Titre : **Etude des régulations du système endocannabinoïde induites par une prise volontaire de cocaïne ou de sucre dans les régions cérébrales associées à la récompense**

Unité de Recherche : **UMR 7364, Laboratoire de Neurosciences Cognitives et Adaptatives**

Directeur de Thèse : **Befort Katia – Chargé de recherche**

ED 414 – Sciences de la vie et de la santé

Introduction

L'addiction

L'addiction est une maladie multifactorielle faisant intervenir des mécanismes génétiques, neurobiologiques, psychologiques ainsi que des facteurs environnementaux. En Europe, la dépendance à la cocaïne affecte 17% des consommateurs réguliers et est caractérisée par une prise chronique, des modifications neurales à long-termes et par la rechute.

Les troubles alimentaires

Le trouble d'hyperphagie alimentaire ou « Binge eating disorder (BED) » est caractérisée par des épisodes de consommation excessive et incontrôlable de nourriture durant une courte période de temps (Diagnostic and Statistical Manual of Mental Disorders, Fifth edition, DSM-5). Les modifications comportementales et moléculaires induites par le BED pourraient être similaires à celles induites par les drogues comme la cocaïne. En effet, quelques études montrent des cibles communes au niveau du système de récompense entre la cocaïne et la prise de nourriture récompensante, en particulier une augmentation des niveaux dopaminergiques dans le circuit de la récompense. C'est pourquoi, notre étude se concentre sur les régions cérébrales associées à la prise de récompense : Cortex Préfrontal (PFC), Nucleus Accumbens (NAc), Striatum Dorsal (DS), Hippocampe (HPC), Amygdale (AMY), Hypothalamus Latéral (LH), Aire Tegmental Ventrale (VTA) and Noyau Tegmental Rostromédian (RMTg).

Le système endocannabinoïde

De façon intéressante, le système endocannabinoïde (ECS) module la prise de récompense, que ce soit avec la cocaïne, la nourriture ou le sucre. Il est composé de deux récepteurs couplés aux protéines Gi/o, les récepteurs cannabinoïdes 1 (CB1) et 2 (CB2) qui sont la cible de deux ligands endogènes, les endocannabinoïdes (eCBs) (Anandamide (AEA) et le 2-arachidonoylglycerol (2-AG)). Certains de ces effets sur la récompense sont déjà caractérisés. Brièvement, alors qu'un agoniste CB1 (HU-210) promeut la recherche de cocaïne chez le rat, un antagoniste (Rimonabant®) la bloque. Il en va de même pour la prise de nourriture, le composé psychoactif du cannabis, le Δ 9 -THC, augmente la prise alimentaire tandis que le Rimonabant® la diminueà le rôle de CB2dans les conduites addictives n'est étudié que depuis très récemment mais ce récepteur semble aussi important. En effet, des infusions intra-NAc d'un agoniste CB2 (JWH-133) diminue la prise de cocaïne chez le rat. Ainsi, l'activation de ces deux récepteurs auraient des effets opposés.

Implication de mécanismes épigénétiques dans les conduites addictives

L'utilisation répétée de drogues induit des adaptations à long-terme dans le système nerveux central. Parmi ces adaptations, des changements dans la plasticité cérébrale ou de l'expression génique sont observés. Ces régulations peuvent être sous le contrôle notamment de mécanismes épigénétiques. Nous avons décrit ces adaptations dans une revue publiée en 2018 dans le journal Neuroscience and Biobehavioral reviews intitulée « Neuroepigenetics and addictive behaviors: where do we stand? De Sa Nogueira D, Merienne K, Befort K ». Ces

mécanismes épigénétiques induisent des changements à long-terme dans l'expression des gènes sans modifier pour autant la séquence d'ADN. Ils incluent la méthylation de l'ADN, les modifications au niveau d'histones (acétylation, méthylation…) ainsi que l'intervention des micro-ARN.

Objectif

Afin de mieux comprendre les adaptations transcriptionnelles induites lors de conduites addictives, mon travail de thèse s'est articulé en plusieurs chapitres.

- ***Projet 1: Neuroadaptations du système endocannabinoïde induites par la cocaïne dans les régions associées à la récompense***

- ***Projet 2: Neuroadaptations du système endocannabinoïde induites par une prise compulsive de sucre dans les régions associées à la récompense***

- ***Projet 3: Neuroadaptations du système opioïde induites par la cocaïne dans les régions associées à la récompense***

- ***Projet 4: LSP29166, un nouvel agoniste orthostérique des récepteurs mGlu4 and mGlu7 réduisant l'auto-administration de cocaïne dans une tâche de ratio progressif chez le rat***

- ***Projet 5: L'activation du récepteur CB2 induit l'expression des facteurs épigénétiques Mecp2 et HDAC2 dans le corps et coquille du noyau accumbens et striatum dorsal***

Je résume ici les deux premiers projets. Pour étudier les modifications de l'ECS induites par une prise volontaire de cocaïne ou de sucre au niveau des régions cérébrales associées à la récompense, nous avons utilisé un modèle d'auto-administration de cocaïne ainsi qu'un paradigme modélisant la prise excessive de sucre chez le rat. Nous avons mesuré les niveaux d'expression des gènes (qPCR) et des ligands endogènes (Spectrométrie de masse) composant l'ECS.

Résultats

Prise volontaire de cocaïne ou sucre

Notre objectif était d'abord d'établir une prise volontaire et chronique de cocaïne ou de sucre. Pour la cocaïne, les animaux s'auto-administrent la cocaïne pendant 10 jours (0.33mg/kg ; FR1). Les animaux consomment la drogue de manière stable et s'auto-administrent 90 injections par jour tandis que les animaux appartenant au groupe contrôle s'auto-administrent du sérum physiologique (non-renforçant). Pour le modèle de consommation de sucre (collaboration Pr MC Olmstead, Canada), les animaux ont accès pendant soit 12h soit 24hr à une solution de sucre (10%) pendant 28 jours. L'accès limité à 12h induit une prise compulsive de sucre dans ce groupe. En parallèle, un premier groupe contrôle n'avait accès qu'à de la nourriture sans sucre tandis qu'un deuxième groupe contrôle avait accès à de la saccharine, laquelle induit une sensation sucrée similaire à celle du sucre mais sans l'apport calorique. Les animaux avec un

accès limité à 12h consomment de manière stable et boivent 2.5x plus de sucre que les autres groupes durant la première d'accès au sucre. Sur une journée entière, la consommation des deux groupes avec accès au sucre est presque similaire, ce qui signifie que le groupe avec accès limité à 12h boit presque autant de sucre que celui avec accès 24h en 2x moins de temps.

Expression des gènes du système endocannabinoïde par la cocaïne ou le sucre

Nos résultats indiquent que la cocaïne modifie significativement l'expression des gènes codants les différents éléments de l'ECS dans un grand nombre de structures cérébrales: PFC, NAc, DS, HPC, AMY, LH, VTA and RMTg. De façon intéressante, l'expression du gène codant pour CB1 est augmentée dans plusieurs structures comme le NAc, DS et HPC. A l'inverse, l'expression de CB2 est quant à elle diminuée dans plusieurs structures comme le PFC, DS et LH. Nous avons aussi mesuré l'expression des enzymes de l'ECS. Leur modulation suggère un changement au niveau de la biodisponibilité des endocannabinoïdes.
En ce qui concerne la consommation de sucre, nos données ne montrent que peu de changements des transcrits du système endocannabinoïdes. Nous observons seulement une augmentation de l'expression de CB1 dans le NAc, similaire à celle induite par la cocaïne.

Modulation des niveaux d'endocannabinoïde par la cocaïne ou le sucre

Comme indiqué précédemment, nos résultats de qPCR suggèrent une modulation des endocannabinoïdes dans le PFC, NAc DS et HPC. Nous avons donc mis au point la mesure de ces ligands lipidiques par spectrométrie de masse (collaboration Dr Y Goumon, INCI, Strasbourg). La cocaïne induit une diminution des niveaux d'AEA dans le striatum (NAc et DS) et à l'inverse une augmentation dans l'HPC. Les niveaux de 2-AG sont augmentés dans le NAc et HPC. La prise compulsive de sucre induit une augmentation d'AEA dans le PFC seulement dans le groupe avec accès limité au sucre (12hr). A l'inverse, nous observons une diminution de 2AG dans ce même groupe dans l'HPC.

Adaptations épigénétiques et chromosomiques induites par la cocaïne dans l'HPC

L'HPC est la structure qui présente les modifications les plus marquées, notamment pour me récepteur CB1. Afin de mieux comprendre ces régulations, nous avons mesuré les modifications de marques d'histones H3K4Me3 et H3K27Ac, toutes deux associées à une augmentation de l'expression des gènes, dans l'HPC. Nous n'observons pas de modifications pour CB1 mais nous notons une augmentation de l'enrichissement de ces marques chez les animaux s'étant auto-administré la cocaïne au niveau d'un gène codant pour une des enzymes de l'ECS., En parallèle, nous avons étudié les potentiels réarrangements chromosomiques au niveau du promoteur du gène codant CB1 dans l'HPC (collaboration Dr K Merienne), et l'analyse de ces résultats est en cours.

Conclusions

De façon intéressante nos travaux mettent en évidence que la prise volontaire et chronique de cocaïne induit de multiples neuroadaptations sur plusieurs niveaux de l'ECS. A l'inverse, la prise compulsive de sucre n'induit que peu d'adaptations sur ce système. Toutefois, nous

observons une adaptation similaire se traduisant par une augmentation de l'expression du gène codant pour CB1 dans le NAC. Etant donné l'importance majeure de cette structure dans les processus addictifs et le rôle de CB1, cette modification indique que la prise compulsive de sucre pourrait induire des mécanismes adaptatifs similaires à ceux induits par la cocaïne et donc causer une dépendance. Etant donné la nécessité de résumer mes travaux de thèse dans ce rapport je n'ai pu décrire l'analyse transcriptomique du NAc que nous avons réalisé dans le NAc des rats consommant du sucre de manière chronique. Brièvement, en utilisant une analyse de priorisation de gènes candidats dans l'addiction, cette analyse indique des activations de processus ou voies similaires à celles induites dans l'addiction à la cocaïne, alcool et nicotine.

Nos résultats de mesure des endocannabinoïdes après une prise de cocaïne ou de sucre n'indiquent quant à eux aucunes similarités. Cela dit, leur modulation intervient dans des structures jouant un rôle majeur dans l'anxiété ou le comportement social, respectivement PFC et HPC. Ces adaptions suggèrent elles aussi un lien envers les comportements addictifs.

En conclusion, nos travaux permettent une meilleure compréhension des processus neuroadaptatifs intervenant dans l'addiction et comportements compulsifs. En particulier, nous mettons ici en lumière des régulations similaire entre la cocaïne et le sucre ainsi qu'une implication majeure de l'ECS dans processus addictifs. Ces travaux devraient permettre d'identifier de nouvelles cibles thérapeutiques afin de pouvoir traiter les patients avec des conduites addictives.

Publications

- The ventral midline thalamus contributes to strategy shifting in a memory task requiring both prefrontal cortical and HPCocampal functions. Cholvin T, Loureiro M, Cassel R, Cosquer B, Geiger K, De Sa Nogueira D, Raingard H, Robelin L, Kelche C, Pereira De Vasconcelos A, Cassel JC. **J Neurosci 2013**

- Neuroepigenetics and addictive behaviors: where do we stand? De Sa Nogueira D, Merienne K, Befort K. **NBBR 2018**

- Cocaine self-administration impacts on the endocannabinoid system: a focus on HPCocampal CB1 receptors and gene chromosomal interaction. De Sa Nogueira D, Filliol D, Romieu P, Chavant V, Goumon Y, Befort K **in preparation**

- Compulsive sucrose intake impacts the endocannabinoid system: clues towards sucrose addiction. De Sa Nogueira D, Filliol D, Chavant V, Goumon Y, Olmstead MC, Befort K **in preparation**

- Cocaine-induced modulation of the rat brain opioid system: a role for HPCocampal mu opioid receptor? De Sa Nogueira D, Filliol D, Romieu P, Befort K **in preparation**

- Activation of cannabinoid CB2 receptor induces expression of the epigenetic factors Mecp2 and HDAC2 in rat striatum. Tesone-Coelho C, De Sa Nogueira D, Filliol D, Munch M, Pol Bodetto S, Zwiller J, Befort K **in preparation**

Posters

- Cocaine-induced regulations of the endocannabinoid system in reward-related brain regions. De Sa Nogueira D, Filliol D, Fonteneau M, Romieu P, Anglard P, Zwiller J, Befort K. INRC 2015. **Poster**

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Présentations

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- Ma thèse en 180 secondes 2017. De Sa Nogueira D **Présentation**

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Voluntary cocaine or sugar intake induce neuroadaptations of the endocannabinoid system in reward-related brain regions

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Résumé

Les troubles alimentaires et l'addiction sont deux pathologies complexes induisant des neuroadaptations à long terme. Dans ce contexte, nous avons étudié les altérations induites par la consommation volontaire de sucre ou de cocaïne dans les régions cérébrales associées à la récompense. Nous avons concentré nos recherches sur les systèmes endocannabinoïde et opioïde, car tous deux sont exprimés dans le système nerveux central et jouent un rôle crucial dans la prise de drogue et de nourriture. Dans l'ensemble, nos résultats mettent en évidence l'hippocampe comme une région cérébrale très impliqué après la prise volontaire de cocaïne. De plus, nos travaux indiquent que certains mécanismes épigénétiques régulent le système endocannabinoïde. Enfin, nous avons pu démontrer qu'une consommation excessive de sucre induit des adaptations transcriptionnelles similaires à celle induites par la cocaïne dans le noyau accumbens. Ainsi, ces résultats pourraient ouvrir la voie vers de nouvelles cibles thérapeutiques pour le traitement des troubles alimentaires ou comportements addictifs.

Addiction ; trouble alimentaire ; système endocannabinoïde ; système opioïde ; épigénétique

Résumé en anglais

Occidental countries currently face an epidemic of obesity and related diseases. As eating disorders and drug addiction are both complex pathologies inducing long-term neuroadaptations, we investigated common alterations induced by either sugar or cocaine intake in reward-related brain regions. We focused our research on the endocannabinoid and opioid systems, as both systems are expressed in the central nervous system and play a crucial role in drug reward and food intake. Overall, our results highlight the hippocampus as a highly involved brain site following cocaine use. Moreover, our work sheds light on epigenetic mechanisms regulating the endocannabinoid system. More importantly, we demonstrate that a binge-like intake of sucrose induced similar transcriptional adaptations to that of voluntary cocaine intake in the nucleus accumbens. These findings may pave the way to new therapeutic targets for addictive behaviors.

Addiction ; Binge-eating ; Endocannabinoid system ; Opioid system ; Epigenetic