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CELL SIGNALLING AND NEUROTRANSMITTER DYNAMICS ASSOCIATED TO SPECIFIC BEHAVIOURAL COMPONENTS OF OPIATE ADDICTION AND IMPULSIVITY

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ABBREVIATIONS

2-AG	2-arachidonoyl glycerol
5' TOP mRNA	5’ terminal oligopyrimidine (TOP) mRNA
5CSRTT	5-choice serial reaction time task
AGC	cAMP-dependent, cGMP-dependent and protein kinase C family
AKT1S1/PRAS40	AKT1 substrate 1
AMPAR	glutamate ionotropic receptor AMPA type
AMPK	AMP-activated protein kinase
ANOVA	analyisis of variance
Atg13	mammalian autophagy-related gene 13
AUC	area under the curve
BLA	basolateral amygdala
BSA	bovine serum albumin
CAD	carbamoyl-phosphate synthetase
CASTOR	cellular arginine sensor for mTORC1
CCD	charge coupled device
cDNA	complementary DNA
CeA	central amygdala
CLIP-170	cytoplasmic linker protein of 170 kDa
CPA	conditioned place aversion
CPP	conditioned place preference
CPT	continuous performance task
CRMP-2	collapsin response mediator protein-2
DAGL	diacylglycerol lipase
DAP1	death-associated protein 1
DAT	dopamine transporter
DDT	delay-discounting task
DEPC	diethyl pyrocarbonate
Deptor	DEP containing mTOR-interacting protein
DLS	dorsolateral striatum
DMS	dorsomedial striatum
DMT	dimethyltryptamine
DNA	desoxyribonucleic acid
ECL	electrochemiluminescence
EDTA	ethylenediaminetetraacetic acid
eIF4B	elongation initiation factor 4B
eIF4E	elongation initiation factor 4E
eIF4EBP	elongation initiation factor 4E binding protein
eIF4F	eukaryotic translation initiation factor 4F
FAAH	fatty acid amide hydrolase
FDR	false discovery rate
FIP200	focal adhesion kinase family-interacting protein of 200 kDa
FKBP	FK-506 binding protein
FRB	FKBP-rapamycin binding domain
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase

GATOR	GTPase activating proteins toward Rags
GSK3	glycogen synthase kinase 3
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HI	high impulsive
HIF1A	hypoxia inducible factor 1α
IGF	insulin-like growth factor
IL	infralimbic cortex
IRS2	insuline receptor substrate-2
ITI	inter trial interval
LI	low impulsive
lOFC	lateral orbitofrontal cortex
LPT	long-term potentiation
LTD	long-term depression
MAGL	monoacylglycerol lipase
mEPSCs	miniature excitatory postsynaptic currents
mGlur	glutamate metabotropic receptor
mLST8	mammalian lethal withSec13protein8
Mmu	mus musculus
mOFC	medial orbitofrontal cortex
mPFC	medial prefrontal cortex
mRNA	messenger RNA
mSin1	mammalian stress-activated map kinase-interacting protein 1
mTOR	mechanistic target of rapamycin kinase
mTORC1	mTOR Complex 1
mTORC2	mTOR Complex 2
NAcc	nucleus accumbens
NAPE-PLD	N-acyl phosphatidylethamolamine phpspholipase D
NMDAR	glutamate ionotropic receptor NMDA type
NSAID	nonsteroidal anti-inflammatory drug
OFC	orbitofrontal cortex
p70S6K	ribosomal protein S6 kinase
PCR	polimerase chain reaction
PDK1	3-phosphoinositide dependent protein kinase 1
PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PIP3	phosphatidylinositol (3,4,5)-trisphosphate
PKB	protein kinase B
PKC	protein kinase C
PND	postnatal day
PRAS40	proline-rich Akt substrate 40 kDa
PrL	prelimbic cortex
protor1	protein observed with rictor 1
protor2	protein observed with rictor s
PTEN	phosphatase and tensin homolog
PVDF	polyvinylidene fluoride
Rag	Ras-related GTPase
Raptor	regulatory-associated protein of mTOR
REDD1	Regulated in DNA damage and development 1

Rheb	Ras homolog enriched in brain
Rictor	rapamycin-insensitive companion of mTOR
RIN	RNA integrity number
RNA	ribonucleic acid
Rno	rattus norvegicus
RT-qPCR	reverse transcription quantitative polimerase chain reaction
S6	ribosomal protein S6
SAMTOR	S-adenosylmethionine sensor upstream of mTORC1
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	stantard error of the mean
SGK1	serum & glucocorticoid-induced protein kinase 1
shRNA	short hairpin RNA
sIPSCs	spontaneous inhibitory postsynaptic current
SLC38A9	solute carrier family 38 member 9
SREBP	sterol regulatory element-binding protein
SSRT	stop-signal reaction time
TBC1D7	TBC1 domain family member 7
TFEB	Transcription Factor EB
THC	Δ-9 tetrahydrocannabinol
TSC	tuberous sclerosis complex
TSC2	tuberous sclerosis complex 2
ULK1	unc-51-like kinase 1
UNODC	United Nations Office on Drugs and Crime
UV	ultraviolet
v-ATPase	vacuolar H+-ATPase
vmPFC	ventromedial prefrontal cortex
VTA	ventral tegmental area

ABSTRACT

The study of the mechanisms mediating the habit-forming actions of opiates is witnessing a second golden age in the current times due to the sad pandemic of opiate-related deaths in the United States of America. Even though there are some effective pharmacotherapies for opioid use disorders, an effective treatment able to revert the addictive phenotype in every individual is missing. This situation is derived from two different facts: there are missing parts in our understanding of how opiates alter brain plasticity process to generate and maintain the addictive behaviour and second, there are still unresolved questions regarding the variables mediating the individual vulnerability to opiate addiction. Concerning this, the study of impulsive behaviour is particularly relevant.

The first experimental series of this thesis is aimed at studying the role of the OFC in waiting impulsivity as measured in two different tasks (the 2CSRTT and the DDT). In particular, we focused on the expression of genes related to glutamatergic, GABAergic or cannabinoid neurotransmission in the lOFC or mOFC divisions of this cortical territory. After studying this vulnerability endophenotype, we turned to opiate self-administration, extinction/withdrawal and reinstatement in two different models. First we studied morphine self-administration in Lewis rats under extended access (12-hour sessions) and the extinction of this behaviour (morphine substituted by saline. We analysed the changes in the mTOR pathway (a signalling cascade involved in brain plasticity and protein synthesis) associated to these behaviours in the amygdala, NAcc and prefrontal cortex (in the case of morphine) or exclusively in the amygdala (in the case of heroin). To date, we are unaware of any other study that has used a self-administration protocol to study the effects of opioid exposure on the mTOR signalling network in rodents. We also decided to study the changes in this pathway after extended access (6-hour sessions) to heroin in a self-administration model and after protracted abstinence (1 day versus 30 days of withdrawal from the drug). This preparation generates a time-dependent increase in cue-induced responding known in the literature as “incubation of craving”.

Our results suggest in the first place that there is a dissociation in the role of the lOFC and mOFC in waiting impulsivity as assessed in the 2CSRTT and DDT. Impulsive choice, a form of waiting impulsivity measured in the DTT positively correlated with CB1 receptor gene expression in the mOFC. However, GluA1 AMPA receptor subunit and $GABA_A$ receptor subunit gene expression was lower in the lOFC in animals with high impulsive actions (as measured in the 2CSRTT).

Morphine and heroine self-administration changed some components of the mTOR pathways, but the effects of the drug were restricted to a small set of genes (such

as Rptor or Eif4ebp2 in the case of morphine, or Rictor, Gsk3a, Igfr1 and 2 for heroin) in the amygdala.

In conclusion, impulsive actions and choices seem to be mediated by different territories of the OFC and by different transmitter systems. Moreover, opiate exposure alters the gene expression of some elements of the mTOR pathway in the amygdala with potential impact on the stimulus-reward associations to which this structure is essential.

RESUMEN

En los últimos tiempos, el estudio de los mecanismos que median la formación de hábitos inducida por opiáceos está viviendo una segunda edad de oro debido a la lamentable pandemia de muertes relacionadas con el consumo de opiáceos en los Estados Unidos de América. Aunque existen terapias farmacológicas efectivas para trastornos por uso de opioides, a día de hoy carecemos de un tratamiento capaz de revertir el fenotipo de adicción en todos los individuos. Esto se debe principalmente a dos realidades: En primer lugar, todavía existen muchas lagunas en nuestra comprensión acerca del modo en que los opiáceos alteran los procesos de plasticidad cerebral para generar y mantener las conductas adictivas. Por otro lado, aún quedan muchas preguntas sin resolver acerca de las variables que median la vulnerabilidad individual a la adicción a opiáceos. Acerca de esto, el estudio de las conductas impulsivas es especialmente relevante.

La primera serie de experimentos de esta tesis tiene como objetivo estudiar el papel de la OFC en la impulsividad de espera medida por dos pruebas distintas (2CSRTT y DDT). Nos hemos centrado particularmente en la expresión de genes relacionados con la neurotransmisión glutamatérgica, GABAérgica o cannabinoide en las divisiones lateral y medial de esta área cortical. Tras estudiar este endofenotipo de vulnerabilidad, pasamos a enfocarnos en la autoadministración de opioides seguida de extinción/retirada y recaída en dos modelos distintos. Primero estudiamos la autoadministración de morfina en ratas Lewis en condiciones de acceso extendido (sesiones de 12 horas) y la extinción de esa conducta (sustitución de morfina por suero salino). Después analizamos los cambios en la vía mTOR (una cascada de señalización involucrada en la plasticidad cerebral y la síntesis de proteínas) asociados a esas conductas en la amígdala, NAcc y corteza prefrontal (en el caso de la morfina) o exclusivamente en la amígdala (en el caso de la heroína). A día de hoy no tenemos constancia de ningún otro estudio que haya usado un protocolo de autoadministración para estudiar los efectos de la autoadministración de opioides en la vía mTOR en

roedores. También decidimos estudiar los cambios en esta vía tras la autoadministración de heroína en un régimen de acceso extendido (sesiones de 6 horas) y después de abstinencia prolongada (un día versus 30 días de retirada de la droga). Este protocolo genera un incremento en las respuestas inducidas por claves dependiente del tiempo conocido como "incubación del ansia por la droga" (craving).

Nuestros resultados sugieren en primer lugar que existe una disociación en el papel de la lOFC y la mOFC en la impulsividad de espera medida por 2CSRTT y DDT respectivamente. La elección impulsiva, un tipo de impulsividad de espera medido por la DTT estaba correlacionada positivamente con la expresión génica del receptor CB1 en la mOFC. Sin embargo, la subunidad GluA1 del receptor AMPA y la subunidad GABAA eran mas bajas en la lOFC de los animales con mayor acción impulsiva (medida por la 2CSRTT).

La autoadministración de morfina y heroína afectó a algunos componentes de la vía mTOR, pero esos efectos se restringieron a un grupo pequeño de genes (como Rptor o Eif4ebp2 en el caso de la morfina o Rictor, Gsk3a, Igfr1 y 2 en el de la heroína) en la amígdala.

Como conclusión, las acciones y elecciones impulsivas parecen estar mediadas por diferentes territorios de la OFC y por diferentes sistemas de neurotransmisión. Aparte, la exposición a opiáceos altera la expresión génica de ciertos elementos de la vía mTOR en la amígdala, lo que potencialmente podría impactar en las asociaciones estímulo-respuesta para las que esta área es esencial.

Chapter 1

Introduction, Hypotheses and Goals

1.1. Introduction to substance use disorders

1.1.1. Origins of drug use

For millennia, humans have been using drugs for recreational, religious or medical purposes (Guerra-Doce, 2015). The first evidence pointing to the use of drugs by humans is a collection of pottery sherds, found in China, with rests of a mixture of a fermented beverage of rice, honey, and fruit, produced as early as in the seventh millennium before the common era (BCE) (McGovern et al., 2004).

The oldest written evidence of medical drug use is an ancient Sumerian tablet written in cuneiform around 2100 BCE. It contained at least fifteen recipes or prescriptions for the preparation and use of ointments and potions mainly from plants with known therapeutic properties (Webb, 1957). We also have evidence of medical drug use obtained from a dead body of a man who lived about 5300 years ago found in the melting ice of the Val Senales glacier (South Tyrol, Italy). After studying the mummified corpse, it was found to be parasitized by *Trichuris trichiura*, a nematode which can infect the human intestine causing abdominal pain and cyclic anaemia (Aspöck et al., 2002). Interestingly, among the belongings of the corpse they were two spheroid masses made from the woody fruit of the fungus Piptoporus betulinus pierced and tied to a leather thong (Figure 1). This fungus has several active compounds with purgative, antibiotic and antiparasitic properties, which could be used to treat digestive conditions, suggesting that "the iceman" was aware of his sickness and was treating it with the available drugs at the moment (Capasso, 1998).

Figure 1: *Detail of the lumps of Piptoporus betulinos presumably used for the treatment of intestinal parasitic diseases. Source: South Tyrol Museum of Archaeology*

In the Lípez highlands of southwestern Bolivia, among other evidence of ancient human occupation, a 1000-year-old ritual bundle was found with several artefacts presumably used for drug administration. The analyses showed rests of psychoactive compounds like cocaine, dimethyltryptamine (DMT), psilocin, bufotenine and harmine, showing extensive botanical knowledge of the owner of the bundle, who probably used it for ritual or healing purposes. (Miller et al., 2019).

1.1.2. Substance use disorders

Unfortunately, there are some drugs that, upon repeated use, can lead to what is commonly known as “addiction”, a cluster of cognitive, behavioural, and physiological symptoms whereby the individual continues using the substance despite significant substance-related problems. This condition is referred to as “substance use disorder” in the fifth edition of the Diagnostic and Statistical Manual of Mental disorders (American Psychiatric Association, 2013). It is difficult to draw a line between controlled substance use and a substance use disorder. For these instances, there are diagnostic criteria (like the example in table 1) used to facilitate the identification and treatment of drug addicts by health practitioners.

Table 1: *Criteria for the diagnosis of substance use disorders. 2-3 symptoms: mild substance use disorder; 4-5 symptoms: moderate substance use disorder; 6 or more symptoms: severe substance use disorder. Symptoms of tolerance and withdrawal are not taken into account if they are expected effects during the course of medical treatment with prescribed medications.*

Criteria grouping	Symptom
Impaired control	The individual may take the substance in larger amounts or over a longer period than was originally intended.
	The individual may express a persistent desire to cut down or regulate substance use and may report multiple unsuccessful efforts to decrease or discontinue use.
	The individual may spend a great deal of time obtaining the substance, using the substance, or recovering from its effects.
	Craving.
Social impairment	Recurrent substance use may result in the failure to fulfil major role obligations at work, school, or home.
	The individual may continue substance use despite having persistent or recurrent social or interpersonal problems caused or exacerbated by the effects of the substance.
	Important social, occupational, or recreational activities may be given up or reduced because of substance use.
Risky use	Recurrent substance use in situations in which it is physically hazardous.
	The individual may continue substance use despite knowledge of having a persistent or recurrent physical or psychological problem that is likely to have been caused or exacerbated by the substance.
Pharmacological criteria	Tolerance.
	Withdrawal.

1.1.3. Trends in drug use

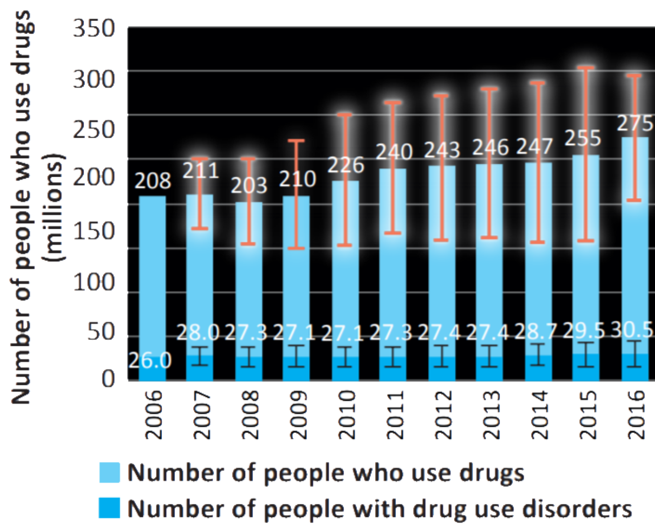


Figure 2: Global trends in the estimated number of people who use drugs, 2006–2016. Source: UNODC, responses to the annual report questionnaire. Note: Estimates are for adults (aged 15–64 years) who used drugs in the past year (United Nations Office on Drugs and Crime (UNODC), 2018).

According to the last World Drug Report (United Nations Office on Drugs and Crime (UNODC), 2018), during the last few years, the number of drug users has been steadily increasing. Moreover, the number of people with drug use disorders increased yearly by one million during the last three years registered (Figure 2). These trends have resulted in an astounding 60% increase in the deaths caused by use of drugs from 2000 to 2015 (Figure 3).

Apart from the deaths and health issues, other addiction-related problems are crime, difficulty in effective functioning in job, family and society in general, economic strain, etc. The impact of these disorders is astounding, but the approach of the government agencies to solve the issue, based on criminalisation and prosecution of producers, dealers and consumers (the infamous war on drugs) has reported little to none effectiveness (Godlee and Hurley, 2016). The resultant stigmatisation of these measures discourage abusers from finding help in health services, and frequently, the most policed

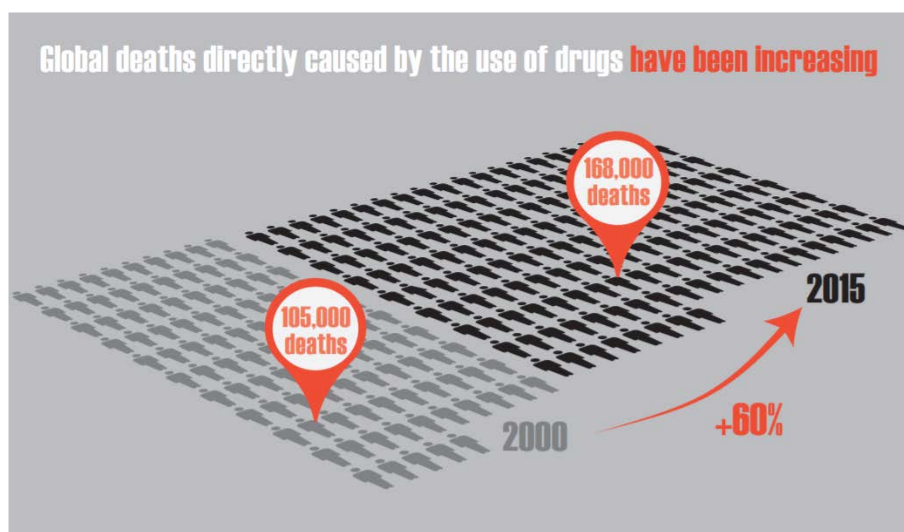


Figure 3: Visual representation of the number of deaths attributable to drug use in 2000 vs 2015. Source: UNODC analysis based on WHO, Disease burden and mortality estimates, Global Health Estimates 2015: deaths by cause, age, sex, country and region, 2000-2015 (United Nations Office on Drugs and Crime (UNODC), 2018)

individuals are the least favoured ones (Csete et al., 2016). Additionally, the legal classification of the drugs is frequently not based on their safety or their addictive potential based on scientific research, and that misleads the users to think that some drugs are safer, even when it is not the case (Nutt et al., 2007).

1.1.4. The case of opioids

Opioids are the drugs which act through opioid receptors. They are named after opium, an extract from the capsule of the *Papaver somniferum* plant, which active component was isolated at the beginning of the XIX century and named morphine (after Morpheus, the Greek god of sleep and dreams) due to its sleep-inducing properties (Serturner, 1805). Morphine is also a potent analgesic and anaesthetic and has been widely used in the medical field. However, its elevated addictive potential is one of its main downsides. Apart from morphine, several other opioids (either naturally-occurring, semi-synthetic or synthetic) have been discovered and used therapeutically or recreationally, several of them having a similar problematic. Some known examples of those addictive opioids are heroin (Daly, 1900), oxycodone (Siegfried, 1918) or fentanyl. (Stanley, 1992). The fact that opioids are very good analgesics commonly prescribed has led to the development of an opioid epidemic crisis, more pronounced in the United States of America, accompanied by an alarming increase in morbidity and mortality associated to opioid use (Kolodny et al., 2015; Volkow et al., 2014)

During 2016, opioids were the drugs which disorders caused most of the deaths attributable to drug use, followed by psychostimulants like cocaine or amphetamine. Moreover, most of the deaths were actually attributed to contagious illnesses frequently transmitted by sharing hypodermic needles for intravenous drug administration, a habitual pathway among opioid users (Figure. 4).

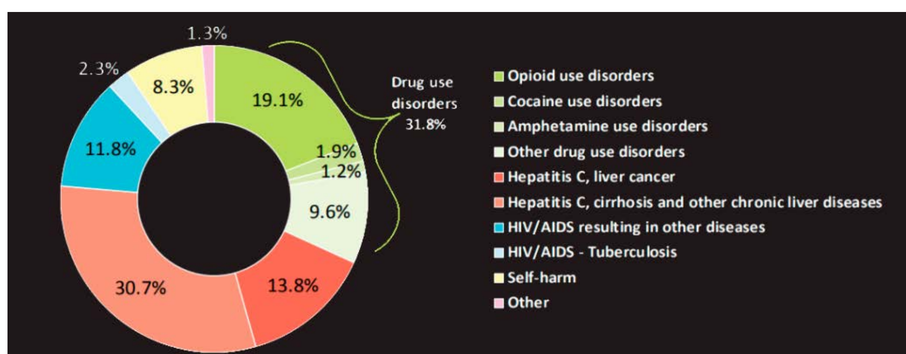


Figure 4: *Leading causes of death attributable to drug use, 2016. Adapted from World Drug Report 2018 (Gakidou et al., 2017; United Nations Office on Drugs and Crime (UNODC), 2018).*

In Spain, the consumption of opioids has decreased over the last years, as reflected in the proportion of entries in urgent care services related to opioid

consumption (Figure. 5A). Despite this reduction, opioid use in Spain is still the first cause of death by acute exposure to drugs, being present in 63.9% of these deaths (Figure. 5B).

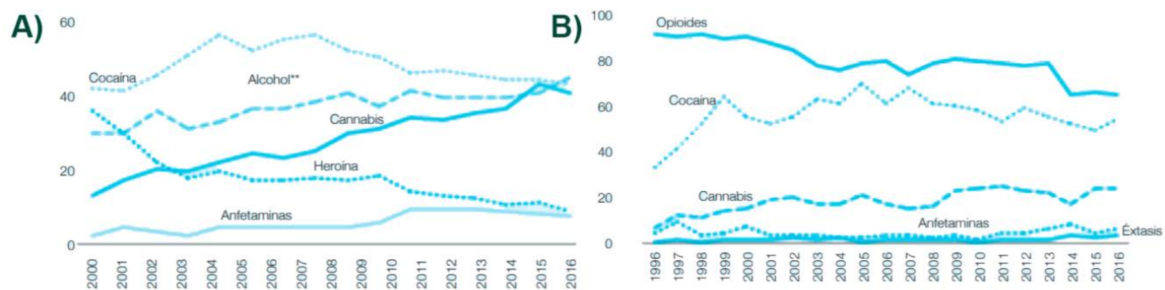


Figure 5: A) *Evolution of the weight of each drug behind the total entries in the Spanish urgent care services related to drug use.* ***B)*** *Percentage of deceases in Spain where each substance is detected among the total deceased as a result of acute intoxication after consumption of psychoactive substances. (Observatorio Español de las Drogas y las Adicciones, 2019)*

1.1.5. Neurobiology of substance use disorders

In 1954, James Olds and Peter Milner published an amazing discovery. Electrical stimulation of several areas of the rat brain had behavioural effects similar to primary rewards like palatable food (Olds and Milner, 1954). Years later, this was replicated in humans (Heath, 1963), and several brain areas and pathways related to this phenomenon were identified. One of those pathways, the mesolimbic one, seemed to have a key role.

The mesolimbic pathway originates in the dopaminergic somas of the VTA and mainly projects to the NAcc, but also to the amygdala, bed nucleus of *stria terminalis*, lateral septal area and lateral hypothalamus (Gardner and Ashby, 2000). This pathway was found to be activated by natural rewards eliciting an increase in dopamine release in the NAcc, which led the research community to refer to this pathway as the reward circuit.

In the following years, it became clear that virtually all addictive drugs evoke an increase in mesolimbic dopaminergic signalling in the NAcc (Bozarth and Wise, 1983; Di Chiara and Imperato, 1988; Irifune et al., 1991; Kuhar et al., 1991; Pontieri et al., 1996; Roberts et al., 1980; Schulteis and Koob, 1996; Wise, 1996; Yoshimoto et al., 1992). Since then, there was a surge in the search for knowledge about the role of dopamine in motivated behaviour, with much progress being made in elucidating the role of this neurotransmitter not only in reward but in associative learning. Decades later, it is still common to see in the media misleading information such as that dopamine is the neurotransmitter of "pleasure" or that addicts are just low on

dopamine because of the adaptations induced by the drugs of abuse they have consumed. It is clear now that mesolimbic dopamine does not just elicit "reward" in the brain but that it actually encodes reward prediction-errors (i.e. the difference between the received and predicted reward) (Schultz et al., 1997). Moreover, the NAcc does not seem to be just a reward area, it is a key region in action selection, integrating cognitive and affective information to increase the efficiency of aversively or appetitively motivated behaviours, especially under ambiguous circumstances (Floresco, 2015).

All this evidence notwithstanding, the mesolimbic pathway has, indeed, an important role in the development of substance use disorders, especially in the initial phases. However, it is an accepted fact that there are many more mechanisms involved in addiction than just reward: failure of control over maladaptive incentive habits (Belin et al., 2013; Everitt and Robbins, 2016), impaired inhibitory control (Goldstein et al., 2009; Goldstein and Volkow, 2011), dysregulation of key neurochemical elements involved in the brain stress systems (Koob et al., 2014), or the impaired salience attribution system (Robinson and Berridge, 2003) are just but a few examples.

There are still some grey areas of vital importance regarding different points along the course of substance use disorders that need further clarification:

In the first place, very little is known about the risk or protective factors which render individuals more or less vulnerable to develop a substance use disorder. The proportion of drug users or laboratory animals developing an addictive disorder varies depending on the drug, but also on genetics and environmental factors. By studying traits associated with higher risk basic researchers may aid clinicians and preventive scientist to develop tailored strategies with increased efficacy.

Secondly, we still do not know what changes take place in the brain when a flexible, goal-directed behaviour such as controlled substance use becomes compulsive. This gap in the knowledge may be filled by using animal models capable that emulate this sort of compulsive drug use.

Finally, one of the defining features of these disorders is the elevated rate of relapse. A 52% of the entries in specialised treatment for cocaine addiction were recidivist patients, and this goes up to 83% in the case of heroin treatments (European Monitoring Centre for Drugs and Drug Addiction, 2019). Understanding how relapse to drug consumption is triggered even in long-term abstinent individuals is a vital point in the development of effective treatments.

The experiments undertaken in this Thesis were aimed at addressing these grey areas and hopefully shedding some light on some unresolved issues.

1.2. Impulsivity and addiction

1.2.1. Impulsivity as a vulnerability trait

The vulnerability to addiction varies among individuals, even under similar drug histories. Similarly to any other condition, there are environmental and genetic factors involved which can either increase the risk of developing a substance use disorder or have a protective effect against it. Understanding the psychobiological mechanisms of this individual vulnerability may result in the design of better strategies for the identification of individuals at risk and also to identify which specific sub-processes should be targeted in an addiction treatment set-up. This has led researchers to look for traits associated with elevated proneness to addictive disorders. One of the identified traits that has been consistently linked to the risk for addiction is impulsivity, a multifaceted construct commonly defined as the tendency to act without foresight, and that depends on both genetic and environmental factors (Bouchard, 2004). The capacity to make rapid (or risky) decisions and act quickly without hesitation can be beneficial in many situations. However, when this tendency becomes extreme it can be detrimental and symptomatic of several psychopathological conditions such as attention deficit hyperactivity disorder or substance abuse (Dalley and Robbins, 2017). Although the neurochemical basis of impulsivity could vary between species, there is evidence of shared mechanisms, at least among mammals. For example, a low turnover in brain serotonin is associated with impulsivity in humans, monkeys and rats (Soubrié, 1986). There is evidence of a link between impulsivity and substance use disorders both in humans (Dom et al., 2006; Ersche et al., 2010; Morris et al., 2016) and animal models (Belin et al., 2008; Dalley et al., 2007; Diergaarde et al., 2008), suggesting that impulsivity can predict the individual vulnerability to substance use disorders.

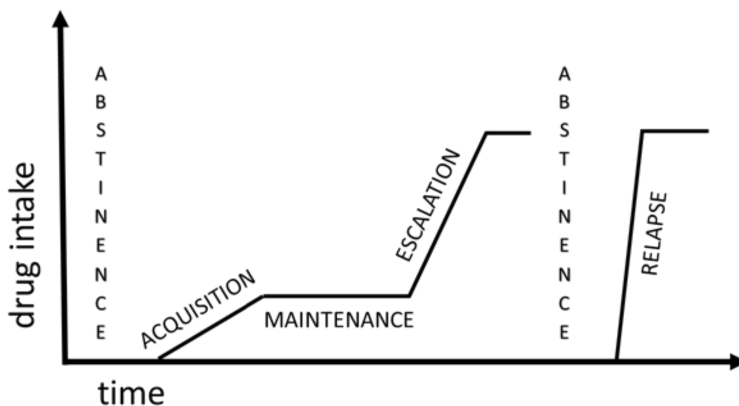


Figure 6: *Stages of substance use disorders. Impulsivity can have a worsening effect during each phase or can trigger the switch to the next stage (Adapted from Perry and Carroll, 2008)*

1.2.2. Varieties of impulsivity

There are several, and some times very different, definitions of impulsivity. It is clear now that impulsivity is not a unitary trait and that it is dependent on different psychobiological mechanisms. These are the most common measures impulsivity both in humans and animal models:

Delay discounting: this form of impulsivity becomes apparent when a small, immediate reward is preferred over larger, but more delayed rewards. This form of impulsiveness is tested in both humans and animal models by observing how the individual switches the preference for each reward as the delay for the bigger reward progressively increases. Impulsive individuals tend to switch earlier to the more immediate reward despite the loss in net reward value.

Probabilistic discounting: based on risk assessment during decision-making, this form of impulsivity is similar to the delay discounting task, but in this case the dependent variable is the probability of obtaining the reward, instead of the delay in reward availability. Impulsive individuals tend to prefer small, certain rewards over larger, but less likely rewards.

Stop-signal reaction time (SSRT): this form of impulsiveness is based on the ability to stop an already initiated action. In the task designed to measure this variety of impulsivity, individuals must perform a task/response. After extensive training, they are occasionally shown a cue that prompts them to make a stop, no-go, response. Impulsive individuals are less capable of restraining their responding after the apparition of the stop signal (Logan, 1994).

Premature responding: this variety of impulsivity is based on the ability to inhibit a response before a waiting interval has elapsed. Subjects (animals and humans) are tested using behavioural paradigms originally designed to assess attentional processes. In the case of humans, several versions of the continuous performance task (CPT) are used (Riccio et al., 2002). In rodents, the most common test is the 5-choice serial reaction time task (5CSRTT) (Robbins, 2002). In both tasks individuals have to respond under certain conditions and punishment (time out, without reward availability) is introduced when the response is premature (made before a *go* signal is presented). The inter-trial interval (time elapsed before the *go* signal is introduced) can be manipulated to stimulate the appearance of premature responses and to better stratify a certain population according to their impulsivity trait. Impulsive individuals are more prone to make premature responses, especially under unexpectedly long inter-trial intervals.

Even though these tasks may be thought to measure the same trait, they are likely to measure different facets of impulsivity with shared and independent mechanisms. Impulsivity measured by the delay discounting task or premature responding do not always correlate with each other (Solanto et al., 2001; Van den Bergh et al., 2006; Winstanley et al., 2004a). For decades, researchers have tried to define the different varieties of impulsivity and identify which one of them is measured in each task. An example of one of these attempts is the segregation of impulsivity in cognitive impulsivity or "impulsive choice" and motor impulsivity or "impulsive action". The tasks involving reward-based decision making (discounting tasks) are regarded as measures of cognitive impulsivity, and the tasks involving motor inhibition (SSRT, 5CSRTT & CPT) would be regarded as measures of motor impulsivity. Considering the last decade of research and on the grounds of the neuroanatomical circuits essential to each test, impulsiveness can also be categorized as "waiting impulsivity" (measured with the delay-discounting task and the 5-CSRTT), "stopping impulsivity" or the difficulty to stop an already initiated action (go/no-go tasks) and the preference for uncertain but bigger outcomes, known as "risk" or "cognitive impulsivity" (probability discounting tasks). Although all these kinds of impulsivity share some common neural mechanisms they also rely on independent pathways (for an excellent review read Dalley and Robbins, 2017).

1.2.3. Neurobiology of waiting impulsivity

Waiting impulsivity seems to be more relatable to substance abuse, as an individual chooses between an immediate small reward (a dose of the drug) over a greater, but more delayed reward (a life free of the burden of addiction). Also, premature responding might reflect how an individual takes more doses than intended, or how easy for impulsive individuals is to relapse. In contrast, stopping impulsivity does not seem to have a relevant relationship with substance use disorders. In addition, the two subtypes of waiting impulsivity predict different aspects of drug addiction (Belin et al., 2008; Diergaarde et al., 2008).

Concerning their neurobiological mechanisms, discounting tasks and premature responding are both mediated by the NAcc. The capacity of delaying gratification is apparently more dependent on the core, while the inhibition of premature responses relies on the integrity of the shell (Basar et al., 2010). Concomitantly, most of the structures with projections to the NAcc are involved at some level in impulsive behaviour. For example, premature responding is mediated by structures like the infralimbic cortex (Chudasama et al., 2003; Murphy et al., 2005), the OFC (Chudasama et al., 2003), insula (Belin-Rauscent et al., 2016), the ventral hippocampus (Abela et al., 2013; Chudasama et al., 2003; Donnelly et al., 2015; Murphy

et al., 2005) and the cingulate cortex (Dalley et al., 2002; Muir et al., 1996). As regards temporal discounting, some of the involved structures are the basolateral amygdala (Winstanley et al., 2004b), the hippocampus (Abela and Chudasama, 2013; Cheung and Cardinal, 2005) and the OFC (Abela and Chudasama, 2013; Kheramin et al., 2002, 2004; Mar et al., 2011; Mobini et al., 2002; Winstanley et al., 2004b; Zeeb et al., 2010). The role of the OFC in delay discounting seems clear, as single units have been found in this area showing increased activity in response to rewards after a long delay independently of the size of the reward (Schoenbaum et al., 2003).

The concrete role of the OFC in waiting impulsivity remains elusive. This elusiveness could be a consequence of the functional dissociation of the lateral and medial OFC shown both in humans (Elliott et al., 2000; Sescousse et al., 2010) and primates (Noonan et al., 2010). In rodents, the study of Mar et al., 2011 revealed a similar functional dissociation between the lOFC and the mOFC. The lesions in the lOFC elicited an increase in waiting impulsivity in a delay-discounting task (DDT) whereas lesions of the mOFC caused the opposite effect. It may be tempting to speculate that this orbitofrontal dissociation could be related to the aforementioned segregation of functions between the core and shell of the NAcc, however, it seems that in the rat (contrary to the monkey) the NAcc is almost devoid of proper orbitofrontal connections (only the lateral portions of the shell receive some projections from the lOFC (Schilman et al., 2008).

Most of the previous studies regarding impulsivity and the OFC have focused on neurotransmitters such as dopamine and serotonin (Dalley et al., 2008; Winstanley et al., 2006), while studies addressing the glutamatergic and GABAergic systems are lacking. Importantly, these transmitter systems have a more direct relationship with the excitation or inhibition status of the region where they are being expressed and little is still known about their roles in impulsivity. In addition, there are several unresolved questions regarding the relationship between waiting impulsivity and the endocannabinoid system, which plays a key role in the modulation GABA and glutamate release from the presynaptic terminals.

1.3. Relapse into substance use

1.3.1. The problem of relapse

One of the central problems regarding substance use disorders is the elevated levels of relapse even after long periods of abstinence (Hunt et al., 1971). In humans, relapse is usually triggered by intense drug craving. This craving can be precipitated y four classical triggers: re-exposure to the drug (de Wit and Stewart, 1981; Jaffe et al.,

1989), drug-associated contexts or cues (O'Brien et al., 1992), stress (Sinha, 2001; Sinha et al., 2011) or withdrawal symptoms (Wikler, 1948, 1973). In the absence of the drug, basal craving and withdrawal symptoms peak early and, as expected, gradually decreases at a rate which depends on the drug. On the other hand, craving induced by drug-related cues is weak after withdrawal and steadily increases over time, an effect seen after withdrawal of cocaine (Parvaz et al., 2016), methamphetamine (Wang et al., 2013), heroin (Wang et al., 2012), alcohol (Li et al., 2015) and tobacco (Bedi et al., 2011). This effect is known as incubation of drug craving, and it has also been modelled in rodents with seeking (extinction) tests at different point of withdrawal (Figure. 7) (Pickens et al., 2011). Understanding why and how this induced craving incubates over time could be crucial for new therapies for relapse prevention.

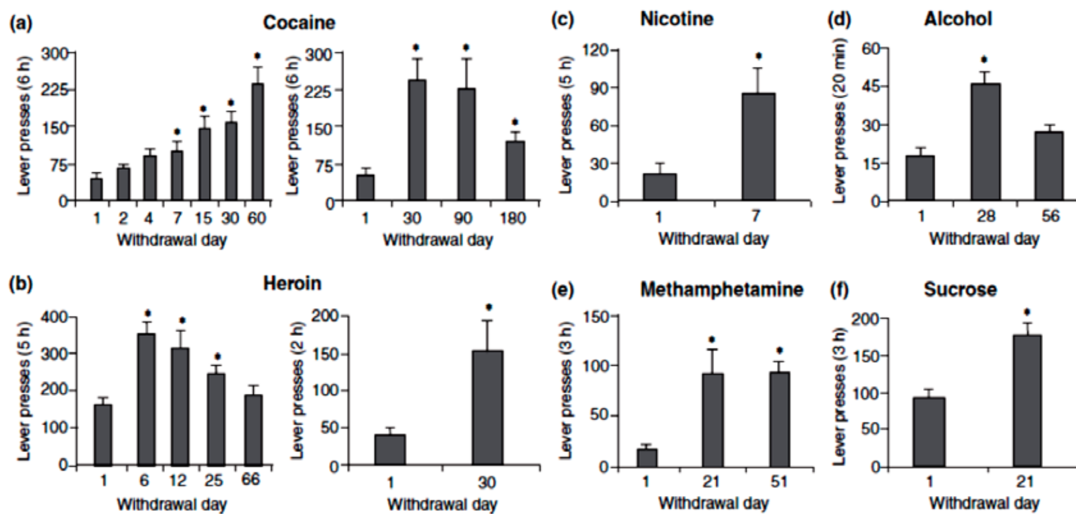


Figure 7: *Incubation of cue-induced drug-seeking along with forced abstinence of different drugs and a natural reinforcer (sucrose) (Pickens et al., 2011):*

1.3.2. Studying cue-induced reinstatement with animal models

1.3.2.1. Extinction based reinstatement models

The concept of extinction takes us back to the famous experiments of Pavlov, who coined the basic concept of classical conditioning with his experiments about the salivary reflexes of the dog (Pavlov, 1927). Pavlov found out that the association of conditioned and unconditioned stimuli could be reversed if the conditioned stimulus is repeatedly presented alone. Later on, Skinner discovered that extinction also applies to operant responding when an automatic pellet dispenser accidentally stopped working and, therefore, operant responding resulted unrewarded (Skinner, 1979). A possible definition of extinction is the disappearance of a previously conditioned response.

The relevance of extinction in addiction treatment is obvious. Cue-induced drug craving is one of the triggers of relapse so, if the association can be extinguished, the rate of relapse could be potentially reduced. Cue-exposure treatment in controlled conditions has been, in fact, used as a therapy for substance use disorders for many years but, at least with the current methods, this therapy has not been very successful when it comes to increasing abstinence in drug-dependent patients (Conklin and Tiffany, 2002).

Box 1: *Extinction*

In extinction-based reinstatement models, rats generally undergo intravenous drug self-administration training in operant conditioning chambers (Fig X). There are different options based on the nature of the drug-associated cues. With discrete cues, each operant response (lever press, nose poke…) is accompanied by a drug infusion paired with a cue (light, tone…)(Davis and Smith, 1976; Meil and See, 1996). Another option is training with the presence discriminative cues (e.g. a specific odour) (Alleweireldt et al., 2001). Also, there is the possibility of using contextual cues, which are the background cues in the environment where the training takes place. When the animal has acquired a stable behaviour, the operant responding is extinguished in the absence of the drug and the cue (the extinction of operant responding associated to contextual cues has to take place in a different environment with another set of background cues). After the extinction, the rodents are subjected to seeking tests, in which the reinstatement is precipitated by re-exposure to the drug-associated cues made contingent to the operant response.

Relapse induced by discriminative cues can also be studied using the operant runway test. For this procedure, the rodent trains to go through a runway maze in the presence of a discriminative cue (e.g. specific odour) and receives a drug dose once it reaches the goal box. The researchers measure how long the rodent takes to reach the goal until it is stable. During the extinction phase, neither the drug nor the discriminative cue is available. During the seeking test the reinstatement is precipitated by the discriminative cue (McFarland and Ettenberg, 1997).

1.3.3. Abstinence based reinstatement models

There are several ways to study reinstatement without relying on extinction training. The easiest way to force abstinence on a subject is by removing access to the drug. Forced abstinence models are easily performed leaving the rodents in their home cages during different withdrawal times. After that, a seeking test can be performed bringing the animal back to the conditioning chamber where operant responding leads to the presentation of the drug-associated cue, but not the drug itself. Researchers register the number of non-reinforced lever presses as a measure of reinstatement of drug-seeking (Reichel and Bevins, 2010). The study of incubation of cue-induced drug-seeking is usually performed comparing cue-reactivity after different lengths of forced abstinence (Pickens et al., 2011).

Although there are scenarios where human addicts undergo forced abstinence (e.g. inmates or patients in rehabilitation centres), most people voluntarily attempt to give up addictions. In an effort to study this phenomenon, some models of voluntary abstinence have been recently introduced. There are two ways to study if rodents would voluntarily refrain from drug-taking: either by associating the drug with an aversive stimulus or by introducing a nondrug reward in a choice procedure. This is also more ecological, as the habitual human motivations for abstinence are either avoiding the consequences of drug abuse or pursuing a more rewarding lifestyle. The aversive stimuli used in animal models are either electric shocks delivered contingently with the drug (In punishment-based reinstatement models) (Panlilio et al., 2003) or an electric barrier around the response detector (in conflict-based reinstatement models) (Cooper et al., 2007). In the models of voluntary abstinence by choice of a nondrug reward the animals have training to self-administer the drug and also palatable food pellets, each with its own associated discrete and discriminative cues. After that, the rodent goes through discrete choice sessions where both discriminative stimuli are shown, and the paired levers are inserted. The animal has to choose one of the levers, and get the associated reward along with the discrete cue, and then both levers are retracted. The intertrial intervals have to be long enough to prevent drug or food satiety, and to avoid anorexigenic/orexigenic effects of the drug (Caprioli et al., 2015b). Interestingly, incubation of cue-induced drug-seeking has also been shown in rats under this voluntary abstinence protocol (Caprioli et al., 2015a; Venniro et al., 2018)

1.4. The mTOR intracellular signaling network and the behavioural constituents of addiction

1.4.1. The importance of intracellular signalling

Addictions and virtually any other kind of behavior be it normal or pathological, could be regarded as the result of specific alterations in the ultra-complex network of communication systems in the body. By virtue of these networks, cells can communicate with each other through myriads of hormones, neurotransmitters and receptors, all of which constitute a central focus in the field of Neuroscience. However, within the cells resides another network that rivals with, if not surpasses, the complexity of intercellular communication. This intracellular network, which regulates all the activity and physiology of the cell, is mostly composed of proteins acting as relays, messengers, adaptors, amplifiers, transducers, integrators, bifurcators and gene expression regulators (Alberts et al. 2002). In acknowledging the key role of intracellular communication cascades in the regulation of every activity of the cell, researchers have spent a considerable amount of time and effort studying the implication of important signaling networks in the actions of drugs of abuse and addictive behaviors. These efforts will hopefully result in a better understanding of each of the pieces that conform the complex puzzle of addiction, in hopes of a better understanding of this psychopathology and perhaps will lead to the development of more effective treatments.

In this work, we focus on a critical node of the intracellular communication system known as the mTOR network. It is named after the mechanistic target of rapamycin (mTOR), a serine/threonine kinase, which interacts with several other proteins to form two functional complexes, the mTOR Complex 1 (mTORC1) and the mTOR complex 2 (mTORC2), with vital functions in key cellular processes such as protein synthesis, cell metabolism, autophagy and neuronal plasticity.

1.4.2. The discovery of mTOR

The discovery of this pathway goes back to the winter of 1964, when the HMSC Cape Scott and her crew departed in an expedition to Easter Island with the ambitious goal of cataloging the health and medical histories of the entire population, the island animals' health, the soil and food sources and the islanders' socio-economic background (Tector 2014). Years later, in 1972, some researchers found a bacterium

Figure 8: *Commemorative seal issued by the Medical Expedition to Easter Island (METEI) designed by.Neehah Molson*

(Streptomyces hygroscopicus) in one of the soil samples capable of synthesizing a molecule with antifungal and antibiotic properties. The isolated compound was named Rapamycin after Easter Island, known as Rapa Nui by the locals (Vézina et al. 1975; Sehgal et al. 1975; Baker et al. 1978; Singh et al. 1979). Subsequent studies revealed that rapamycin also had immunosuppressant (Martel et al. 1977), and antitumoral properties (Douros and Suffness 1981).

The mechanism of action of rapamycin was not discovered straight away. The first clue was not found until 1990 when a group of researchers observed that rapamycin and another immunosuppressant called FK-506 mutually antagonized their activities (Dumont et al. 1990). It became clear at the time that both molecules bound with similar affinities to an abundant cytosolic protein, which was named FK-506 binding protein (FKBP) (Bierer et al. 1990). Later, more subtypes of FKBP were discovered (Galat 1993), and the rapamycin/FKBP interaction was proven to be necessary for rapamycin to exert its effects (Ocain et al. 1993). At this point, some of the downstream effectors had already been identified (Chung et al. 1992), but the rapamycin-FKBP complex did not seem to activate these effectors by itself. Finally, the target of the complex was detected in yeast extracts and named "target of rapamycin" or TOR (Heitman et al. 1991). When a homologous protein, which also interacted with the rapamycin/FKBP complex, was found in the rat brain and murine T-lymphoma cells (Sabatini et al. 1994; Sabers et al. 1995), it was appropriately named mammalian target of rapamycin or mTOR. However, as mammalian is not transferable across all species, it has recently been renamed as mechanistic target of rapamycin kinase (HUGO Gene Nomenclature Committee, 2019)). Neither rapamycin nor FKBP interacts with mTOR separately but, when associated, they bind strongly to a 133-amino acid domain of mTOR known as the FKBP-rapamycin binding domain (FRB) (Banaszynski et al. 2005; Chiu et al. 1994).

Once the identity of the immediate downstream intracellular partner of the rapamycin-FK596 complex was clarified, the biochemical findings on the components and functions of the mTOR network increased exponentially until the present date. The mTOR protein was found to be associated with others, forming two different functional complexes with different subunits and functions, as mentioned before (see Figure 1). Six components of mTORC1 and seven of mTORC2 have been discovered so far, four of them are shared by both complexes: mTOR, which is the catalytic subunit, the mammalian lethal with Sec13protein8 (mLST8) (Kim et al. 2003), DEP containing mTOR-interacting protein (Deptor) (Peterson et al. 2009) and the Tti1/Tel2 complex (Kaizuka et al. 2010). The mTORC1 is also constituted by the regulatory-associated protein of mTOR (Raptor) (Hara et al. 2002) and the proline-rich Akt substrate 40 kDa (PRAS40) (Sancak et al. 2007; Wang et al. 2007; Haar et al. 2007; Thedieck et al. 2007). mTORC2 on the other hand, is composed of the rapamycin-insensitive companion of mTOR (Rictor) (Dos D. Sarbassov et al. 2004; Jacinto et al. 2004), the mammalian stress-activated map kinase-interacting protein 1 (mSin1) (Jacinto et al. 2006; Frias et al. 2006) and the proteins observed with Rictor 1 and 2 (protor1/2) (Pearce et al. 2007; Thedieck et al. 2007). Rapamycin was found to inhibit only mTORC1 activity due to its inability to bind to mTORC2 (Dos D. Sarbassov et al. 2004; Jacinto et al. 2004). However, it has been observed in further studies that a rapamycin treatment of an adequate duration is able to reduce mTOR levels, an effect probably mediated by mTORC2 assembly inhibition (Sarbassov et al. 2006) by virtue of the capacity of rapamycin to bind to free mTOR (Sabatini et al. 1994; Sabers et al. 1995). mTORC2 inhibition could also be accomplished via complete dephosphorylation and cytoplasmic translocation of nuclear Rictor and mSIN1, a mechanism that is observed after prolonged rapamycin treatments and correlates with the reduction of mTORC2 levels (Rosner and Hengstschläger 2008; Akcakanat et al. 2007).

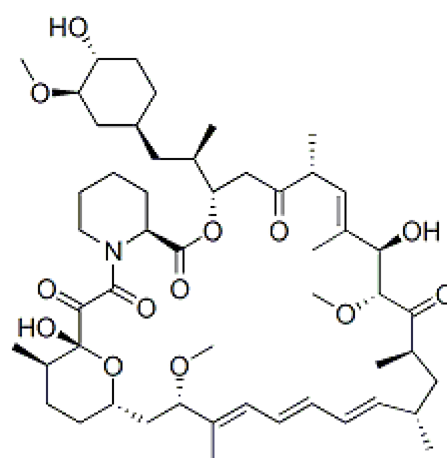


Figure 9: *Skeletal formula of rapamycin*

1.4.3. mTORC1 and mTORC2 control (almost) everything

The perplexing variety of processes under the regulation of mTORC1 and mTORC2 provides an idea of the role of this signaling cascade in the general physiology of the cell and suggests that any deviation from the normal function of the pathway may have dramatic consequences to the organism as a whole. What follows is a brief description of some of the more important functions of the mTOR complexes.

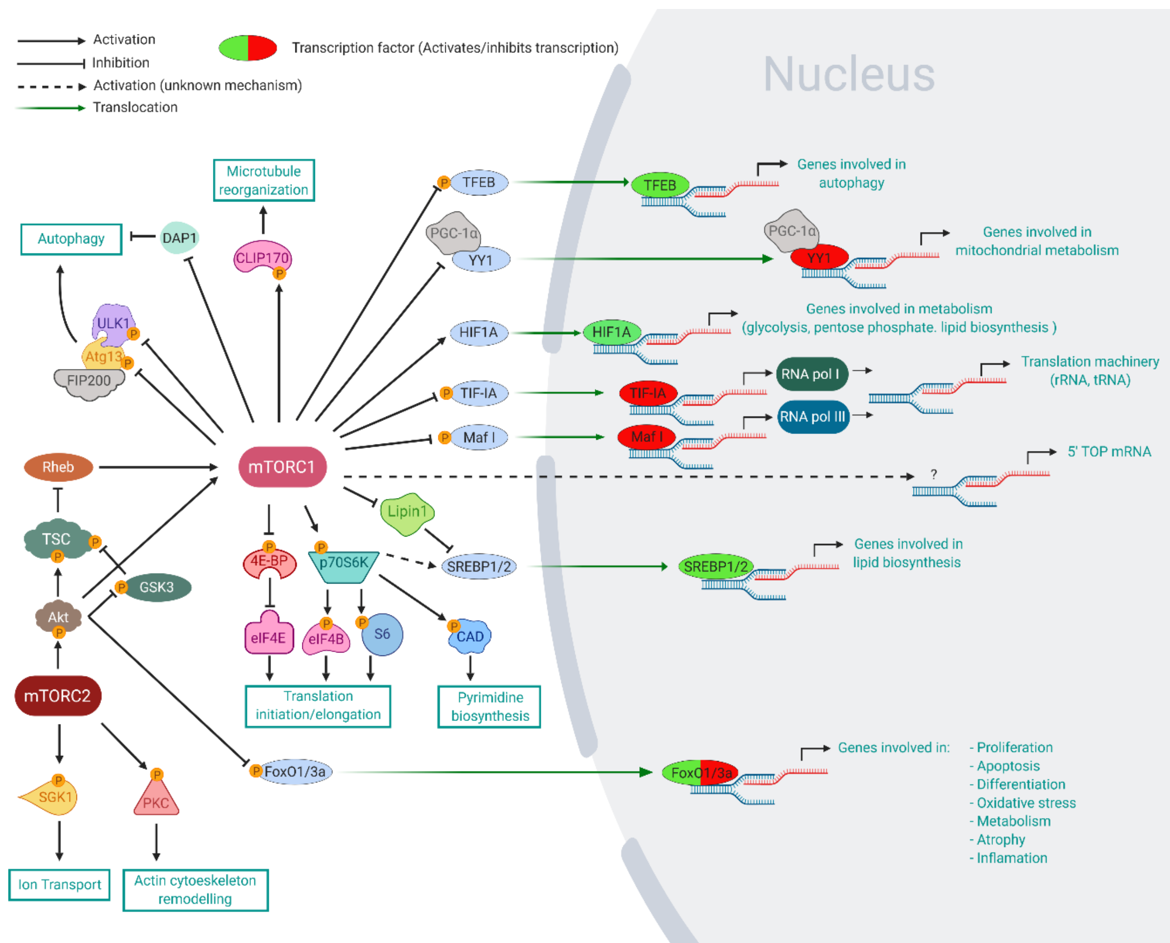


Figure 10: *A graphical summary of the current knowledge of the mTOR network and the cellular processes under the regulation of mTORC1 and mTORC2*

The first known cellular function regulated by mTORC1 was protein synthesis. mTOR regulates protein synthesis employing a wide range of mechanisms. One of them is the eukaryotic translation initiation factor 4F (eIF4F), another protein complex responsible for bridging the mRNA and the ribosome and regulating transcription (for an excellent review about initiation factors and regulation of protein translation see Sonenberg and Hinnebusch, 2009). mTORC1 mediates the phosphorylation of elongation initiation factor 4E binding proteins (eIF4EBP1, eIF4EBP2 & eIF4EBP3). These eIF4EBPs are usually bound to the elongation initiation factor 4E (eIF4E), one of the proteins of the elongation initiation factor 4F (eIF4F) complex, preventing its binding to the rest of the components. (Poulin et al. 1998; Pause et al. 1994). The function of eIF4E is the recognition of the 5'-terminal cap of mRNAs (Sonenberg et al. 1978). When mTORC1 phosphorylates eIF4EBPs, eIF4E is free to join the eIF4F complex allowing the initiation of transcription. mTORC1 also phosphorylates p70S6K, which can regulate protein synthesis phosphorylating the 40S ribosomal subunit protein S6 (S6) and the elongation initiation factor 4B (eIF4B) (Holz et al. 2005). Moreover, mTORC1 also regulates the transcription of a specific kind of mRNAs which

encode most of the protein components of the translational machinery and have an oligopyrimidine tract at the 5′ end (5′ TOP mRNAs). This regulation is not dependent on p70S6K, as previously suspected; the mechanism, however, remains unknown (Tang et al. 2001). In addition, mTORC1 regulates RNA polymerase III transcription phosphorylation and inhibits Maf I, a repressor of RNA polymerase III transcription (Shor et al. 2010) and RNA polymerase I activity by phosphorylating and inhibiting the transcription factor TIF-IA (Mayer et al. 2004), thus controlling the transcription of the translational machinery.

Another process regulated by mTORC1 is autophagy, the primary mechanism of degradation of cellular components. mTORC1 represses autophagy interacting with the kinase complex formed by unc-51-like kinase 1/mammalian autophagy-related gene 13/focal adhesion kinase family-interacting protein of 200 kDa (ULK1/Atg13/FIP200) complex (which generally promotes autophagy). mTORC1 phosphorylates both ULK1 and Atg13, inhibiting them and therefore repressing autophagy (Ganley et al. 2009; Jung et al. 2009; Hosokawa et al. 2009). Also, mTORC1 inhibits the expression of genes related to autophagy and lysosome biogenesis phosphorylating the Transcription Factor EB (TFEB), preventing its translocation to the nucleus (Settembre et al. 2012; Roczniak-Ferguson et al. 2012; Martina et al. 2012). On the other hand, mTORC1 promotes autophagy by inactivating the autophagy repressor death-associated protein 1 (DAP1). This opposing mechanism is hypothesized to be a means for mTORC1 to control a specific balancing brake, and, in so doing, limit the autophagic response, maintaining as a consequence a proper homeostatic balance (Koren et al. 2010).

The third function of mTORC1 is the regulation of cellular metabolism. For example, mTORC1 activity elicits an increase in translation of hypoxia-inducible factor 1α (HIF1A), which in turn promotes the transcription of several genes related to metabolism. As a result, several cellular processes are activated such as stimulation of glycolysis, activation of the oxidative arm of the pentose phosphate pathway, and induction of de novo lipid biosynthesis (Hudson et al. 2002). In addition to this, mTORC1 also stimulates the activity of sterol regulatory element-binding proteins (SREBP1 & SREBP2), which control the expression of genes involved in fatty acid and cholesterol biosynthesis. This regulation is mediated by p70S6K (Düvel et al. 2010) and also by the phosphorylation of a SREBP inhibitor, Lipin1 (Peterson et al. 2011). Moreover, p70S6K has also a role in nucleoside biosynthesis regulation via the phosphorylation of carbamoyl-phosphate synthetase (CAD), a component of the pyrimidine synthesis pathway, which results in its activation (Ben-Sahra et al. 2013; Robitaille et al. 2013). Finally, mTORC1 regulates mitochondrial metabolism by

altering the expression of several mitochondrial genes (Schieke et al. 2006; Cunningham et al. 2007).

Another process involving mTORC1 is microtubule organization. mTORC1 mediates the phosphorylation and activation of cytoplasmic linker protein of 170 kDa (CLIP-170), a protein crucial for microtubule organization and functions (such as dendrite formation, for example) (Choi et al. 2002; Swiech et al. 2011).

In contrast with mTORC1, our knowledge about mTORC2 remains very limited, probably due to the absence of specific mTORC2 inhibitors. Most of mTORC2 substrates belong to the family of the cAMP-dependent, cGMP-dependent, and protein kinase C (AGC) protein kinases (Arencibia et al. 2013). For example, the best studied mTORC2 substrate is the kinase Akt (also known as protein kinase B or PKB), which is a vital node of the mTOR network (Sarbassov et al. 2005). Apart from mTORC2, Akt is activated by the 3-phosphoinositide-dependent protein kinase 1 (PDK1) as part of the PI3K/Akt signaling pathway, which is mainly activated by insulin and growth factors (Alessi et al. 1997) and inhibited by the phosphatase and tensin homolog (PTEN) (Georgescu 2010). Akt regulates cellular processes such as metabolism, survival, apoptosis, growth, and proliferation by phosphorylating substrates such as glycogen synthase kinase 3 (GSK3α/β), the Tuberous Sclerosis Complex 2 (TSC2), AKT1 substrate 1 (AKT1S1 or PRAS40) and theFoxO1/3a transcription factors, among others (Madhunapantula et al. 2011). Another mTORC2 substrate is Serum & Glucocorticoid-induced protein Kinase 1 (SGK1), which regulates diverse effects of extracellular agonists by phosphorylating regulatory proteins that control cellular processes such as ion transport and growth (García-Martínez and Alessi 2008). mTORC2 also regulates cell shape by phosphorylating several members of the protein kinase C (PKC) family, which are involved in actin cytoskeleton remodeling (Gan et al. 2012; Thomanetz et al. 2013; Li and Gao 2014; Dos D. Sarbassov et al. 2004). Due to its involvement in actin polymerization regulation, mTORC2 is required for hippocampal long term potentiation (LTP) (Huang et al. 2013) and long term depression (LTD) (Zhu et al. 2018). Finally, like mTORC1, mTORC2 seems to be involved in autophagy regulation (Lampada et al. 2017) (see Figure 10).

1.4.4. But, who's behind the wheel?

With such diverse effectors, it should be evident that the regulation of the mTOR complexes is also intricate and refined. Just like the previous examples, we have a better understanding of the regulation of mTORC1.

mTORC1 integrates a broad range of both extracellular and intracellular inputs, most of them converging in the tuberous sclerosis complex (TSC), a critical mTORC1

inhibitor. The TSC has three components: TSC1, TSC2 (also known as hamartin and tuberin) (Tee et al. 2003), and TBC1D7 (Dibble et al. 2012), and inactivates the GTPase Ras homolog enriched in brain (Rheb), an mTORC1 activator, inhibiting mTOR in consequence (Long et al. 2005) (see Figure 11).

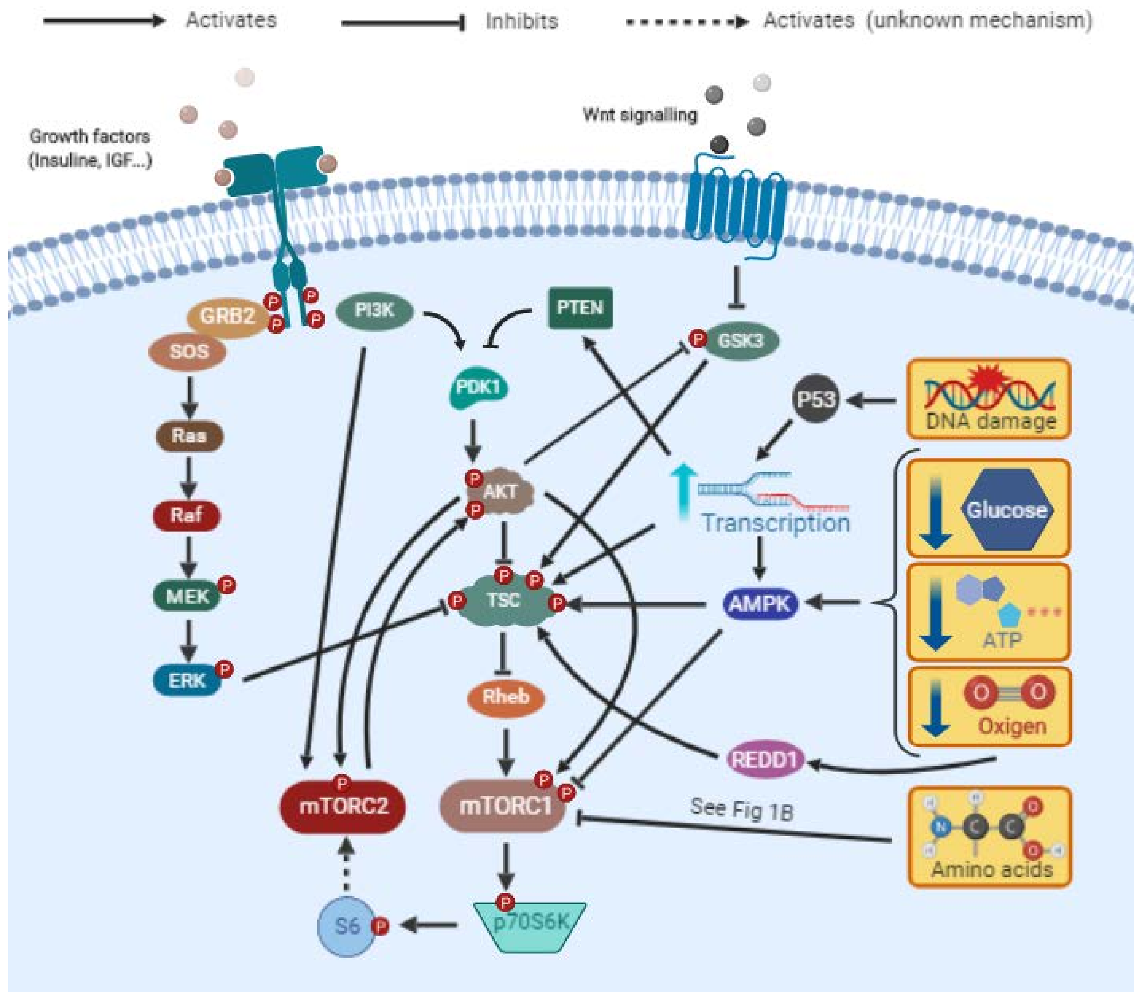


__Figure 11:__ Mechanisms of mTORC1 and mTORC2 regulation

Growth factors like insulin or insulin-like growth factors (IGF1 or IGF2) inhibit TSC activity through two different signaling pathways. They activate the PI3K/PDK1/Akt signaling cascade, which results in the phosphorylation of TSC2 by Akt and therefore, the inhibition of the TSC (Inoki et al. 2002). Growth factors also activate the Ras/Raf/MEK/ERK pathway, which also results in the phosphorylation and inhibition of TSC2 (Ma et al. 2005). Apart from TSC inactivation, as we mentioned when reviewing mTORC2 effectors, Akt phosphorylates and inactivates PRAS40, an inhibitor component of the mTORC1 (Haar et al. 2007; Sancak et al. 2007). Moreover, Akt also disinhibits mTOR by phosphorylating and repressing GSK3, which generally phosphorylates and promotes TSC2 activity (Inoki et al. 2006; Tong et al. 2002). This

regulation through GSK3 inhibition is also shared by the Wnt signaling pathway (Inoki et al. 2006).

Due to its implication in protein synthesis regulation, it is not surprising that mTORC1 activity is also subject to regulation by amino acid levels (Figure 12). There are several mechanisms ensuring that mTORC1 signaling is turned off when amino acids are scarce. For example, intra-lysosomal amino acids are sensed through an unknown mechanism dependent on the vacuolar H+-ATPase (v-ATPase) and the lysosomal membrane protein solute carrier family 38 member 9 (SLC38A9), which interact with a protein complex named Ragulator (Rebsamen et al. 2015; Jung et al. 2015; Wang et al. 2015a; Zoncu et al. 2011). This complex modulates the activity of a group of small GTPases called Ras-related GTPases (Rag), which bind mTORC1 to the lysosomal surface, where Rheb, its activator, is also bound when active (Sancak et al. 2010; Sancak et al. 2008; Bar-Peled et al. 2012). mTORC1 activity is also sensitive to cytosolic amino acids. Cytosolic leucine is sensed by the protein Sestrin2, while arginine is sensed by the cellular arginine sensor for mTORC1 (CASTOR) and methionine by S-adenosylmethionine sensor upstream of mTORC1 (SAMTOR). All of them act through the GTPase activating proteins toward Rags (GATOR) complexes, which also interact with Rag GTPases (Parmigiani et al. 2014; Chantranupong et al. 2016; Saxton et al. 2016; Chantranupong et al. 2014; Gu et al. 2017). Another regulator of Rag GTPases relaying amino acid levels information is the complex Folliculin-FNIP2

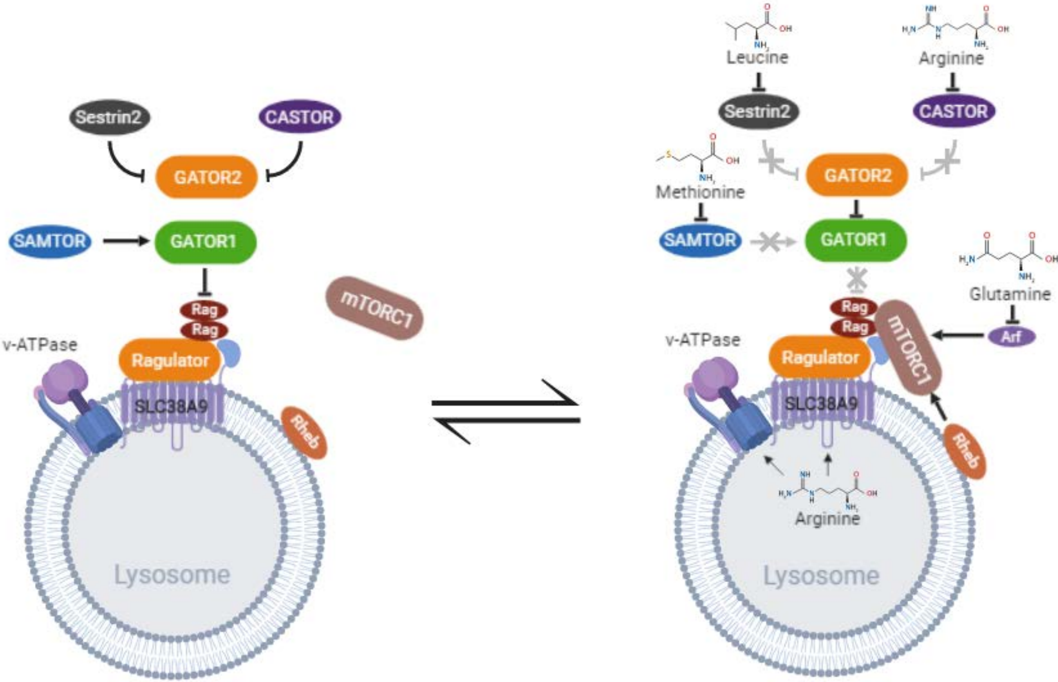


Figure 12: *mTORC1 regulation by amino acid sufficiency*

(Petit et al. 2013; Tsun et al. 2013). On the other hand, a Rag-independent pathway has been discovered for glutamine-sensing, which recruits mTOR through the Arf family GTPases instead (Jewell et al. 2015).

Another repressor of mTORC1 activity is the AMP-activated protein kinase (AMPK), which phosphorylates TSC2 and Raptor under glucose/ATP depletion (Shaw et al. 2004; Inoki et al. 2006) or hypoxia (Arsham et al. 2003). Interestingly, glucose levels also affect mTORC activity through Rag GTPases, proving them not to be amino acid-exclusive sensors (Kalender et al. 2010). Hypoxia also has an alternative pathway to repress mTORC1 by inducing Regulated in DNA damage and development 1 (REDD1), which activates TSC (Brugarolas et al. 2004).

Finally, mTORC1 is also inhibited when DNA damage is detected. The subsequent activation of p53 activates the transcription of the already mentioned mTORC1 repressors TSC2, PTEN, and the regulatory subunit β1 of the AMPK (Feng et al. 2007).

The regulation of mTORC2 is not as well characterized as in mTORC1 but seems to be quite different from that of mTORC1. In contrast with mTORC1, mTORC2 kinase activity is basally autoinhibited by its mSIN1 subunit (Liu et al. 2015). Growth factors elicit an increase in PI3K activity (or a decrease in PTEN activity), increasing the levels of Phosphatidylinositol (3,4,5)-trisphosphate (PIP3), which binds to mSIN1 relieving the inhibition (Liu et al. 2015). Moreover, Akt activity is also increased through PI3K signaling and can also phosphorylate mSIN1 and activate mTORC2 creating a positive feedback since Akt is a substrate of mTORC2 itself (Yang et al. 2015). It has also been observed that mTORC2 activation is dependent on its binding to the ribosome (Zinzalla et al. 2011), which is mediated by S6, one of the downstream effectors of mTORC1 (Yano et al. 2014).

1.4.5. The mTOR network and the reward associated to drugs of abuse

The most obvious conclusion reached after all these discoveries is that the mTOR network is a hub where several processes converge to regulate growth factor-dependent cell maturation and cellular status in general. Consequently, there have been efforts to target the pathway to treat diseases related to uncontrolled cell growth like cancer, with very successful results (Pópulo et al. 2012; Guertin and Sabatini 2007). A less obvious conclusion was that, as a regulator of protein synthesis and microtubule reorganization, mTOR could also be related to synaptic plasticity and neurological disorders. This conclusion was also proved to be true for both mTORC1 and mTORC2 (Hoeffer and Klann 2010). Having a role in synaptic plasticity, the mTOR network could be involved in many behavioral and cognitive processes including, to a

yet to be determined extent, drug addiction (Neasta et al. 2014; Pei et al. 2019; Laguesse and Ron 2019).

Even though addictions share common mechanisms, drugs of abuse act through very different molecular targets and, apparently, they also have different ways to interact with the mTOR network. We have plenty of evidence showing changes in the activity or expression of various elements of the mTOR cascade after different treatment schedules with alcohol (Ben Hamida et al. 2012; Neasta et al. 2010; Neasta et al. 2011; Ben Hamida et al. 2018; Beckley et al. 2016; Laguesse et al. 2017a; Li and Ren 2007), nicotine (Gao et al. 2014; Jin et al. 2008; Tsurutani et al. 2005; Zhang et al. 2007), cannabinoids (Puighermanal et al. 2009; Derkinderen et al. 2003; Rubino et al. 2004; Puighermanal et al. 2013; Blázquez et al. 2015; Shrivastava et al. 2011; Renard et al. 2016), opioids (Russo et al. 2007; Muller and Unterwald 2004; Mazei-Robison et al. 2011; Xu et al. 2014; Wang et al. 2015b; Olianas et al. 2011; Polakiewicz et al. 1998), cocaine (Wu et al. 2011; Miller et al. 2009; Xu et al. 2009; Perrine et al. 2008; Miller et al. 2014; Álvaro-Bartolomé and García-Sevilla 2013), amphetamine (Beaulieu et al. 2004), methamphetamine (Narita et al. 2005; Ma et al. 2014; Kongsuphol et al. 2009; Li et al. 2012; Huang et al. 2018; Xu et al. 2011) and ketamine (Li et al. 2010). Notwithstanding the good understanding of the complex mechanisms behind the modulation of the network by each drug, the most exciting changes are those relative to the reward processes that drugs of abuse enact to initiate the transition to addiction. Addiction and reward are not the same phenomenon. In the last years refined behavioral models have been developed that recapitulate some of the key features of addiction (such as the search of the drug even when it is not available, increase motivation to consume the drug and drug-seeking or taking even in the face of adverse consequences) (for an in-depth review of these behavioral models of addiction, see Belin-Rauscent et al., 2016; Everitt et al., 2018; Sanchis-Segura and Spanagel, 2006 and Spanagel, 2017). With some exceptions (Brown et al. 2011), these sophisticated models of addiction have yet to be applied in the investigation of the involvement of the mTOR pathway in addictive disorders. There are other paradigms however that capture simpler and more elemental constituents of the addictive phenotype (such as primary and conditioned reward, pavlovian context-reward associations, increased locomotor responses after repeated exposure to the drug, etc…). Here we summarize the most

relevant findings in studies using these paradigms (see Box 1 for a succinct description of these models).

Sensitization: Certain acute effects of drugs are dampened (tolerance) or potentiated (sensitization) as a consequence of repeated administration. Rodent models of sensitization are frequently used to study the adaptations behind these phenomena, which are supposed to be similar to some of the adaptations mediating certain features of addictions. Locomotor sensitization to drugs of abuse is typically studied by giving systemic injections (or even a single injection) of the drug to be tested during the so called 'induction' or 'development' phase to experimental animals. Then, in a test session, the acute behavioural responses to a challenge drug injection is compared in rats pretreated with the drug versus the responses of rats without such history (see Vezina, 2004 for a review).

Conditioned place preference (CPP): This paradigm is habitually used to measure the aversive or reinforcing properties of drugs by means of the pavlovian associations that are established between the context in which drug exposure takes place and the rewarding or aversive effects induced by the drug. A conditioning chamber consisting in two compartments with visual (and sometimes tactile) cues is used in CPP experiments. Both compartments are communicated by an alley. The procedure usually starts with a preference tests in which the animal is free explore the two compartments and the center area, in order to look for unconditioned preferences for any of the compartments. This is followed by the conditioning sessions in which the animals are sequentially administered the drug of interest or its vehicle while being confined in either compartment (normally in a counterbalanced order). Finally, the test session/s are performed in a similar way to the preconditioning session, with free-access to both compartments. The preference score is then calculated as the proportion of time spent in the drug-paired chamber as compared to the time spent in the same compartment during baseline (see Bardo and Bevins, 2000 for a review).

Operant drug self-administration: By taking advantage of the principles and technologies of instrumental learning, the voluntary consumptions of drugs can be studied in operant conditioning chambers. In a typical self-administration setting an operant response (typically a lever press or a nose poke) is required to initiate drug delivery. The role of conditioned stimuli can also be studied by pairing cues to the delivery of the drugs. Several schedules of reinforcement can be applied to determine the contingencies between the operant response and drug delivery (ratio schedules –when the reward is administered after a certain, or increasing –progressive- number of responses has been emitted-; interval schedules -the drug is administered when the response is emitted after a given period of time- etc…). Relapse or reinstatement of drug use can be modelled by submitting the animals to an extinction period (in which responses are no longer paired with drug delivery) until response rates decrease to a minimum and the by re-introducing a drug-paired cue (including the context in which the drug was self-administered), exposing the animal again to the drug, or by submitting them to stressors. Under these conditions, the operant response is reinvigorated, a phenomenon that is normally interpreted as drug-seeking. These models allow for a very fine control all the relationships between the stimuli, the responses and the rewards, and have high ecological validity (Everitt et al., 2018; Spanagel, 2017).

Drug-associated memory reconsolidation: When memories are recalled, they are postulated to enter in a labile state into which they can be updated (weakened or reconsolidated). This also applies to drug-related memories, an effect that could take part in the strengthening of memories of drug-associated cues and contexts. Moreover, during that labile state, memories are susceptible to be disrupted by blocking their reconsolidation. This is interesting from a therapeutic point of view, as it could be used to help to wipe out the associations between drug and cues and, in so doing, attenuate drug-taking reinstatement. In order to study and manipulate drug-associated memory reconsolidation it is enough to expose the subject to a previously drug-paired cue under certain conditions. The expression of the reconsolidation can then be assessed under extinction conditions in a CPP or operant responding task, for example (see Exton-McGuinness and Milton, 2018 for a review).

Box 2: *Behavioural tests used in the study of the relevance of intracellular signaling pathways in addiction-related behavioural traits*

1.4.5.1. Sensitization

The development of sensitization to different effects of addictive drugs have been extensively studied as a model of the neuroadaptations mediating addiction (Yamamoto et al. 2013). See Table 1 for a summary of the relevant results of the studies reviewed below.

Psychostimulants have been the most studied drugs in terms of their relationship with the mTOR pathway. Selective mTOR manipulations during the induction or test phases have revealed important information about the specific roles of mTORC1 and mTORC2 in each phase of the phenomenon. For example, it has been shown that a rapamycin pretreatment either during the acquisition sessions or before the challenge test prevents cocaine-induced locomotor sensitization, showing that mTORC1 is necessary for both the development and expression of locomotor sensitization (Wu et al. 2011). However, a similar protocol of rapamycin pretreatment did not affect cocaine-induced locomotor sensitization, although a single dose of rapamycin one hour before the test prevented its expression (Bailey et al. 2012). The inhibition of PI3K during the induction phase did not prevented the expression of cocaine sensitization after the challenge, however, when the inhibitor was administered before the test, the expression of locomotor sensitization was abolished (Izzo et al. 2002). This is proof that that PI3K activity is necessary for the expression of locomotor sensitization, but not for its development. Of note, animals sensitized to the locomotor effects of cocaine have higher phosphorylation levels of PI3K in the nucleus accumbens (NAcc) shell and prefrontal cortex (PFC) (but lower levels in the NAcc core) (Zhang et al. 2006).

During the acquisition phase of a methamphetamine behavioral sensitization protocol in mice, both total and phosphorylated mTOR progressively increases in the NAcc (but not in the striatum) with each daily methamphetamine injections. This protocol also elicited an increase in phosphorylated P70S6K and S6, but not Akt, suggesting that mTORC1 signaling is involved in the induction of methamphetamine sensitization. Rapamycin i.p. administration before the daily methamphetamine induction doses suppressed methamphetamine-enhanced dendritic arborization in the NAcc. Also, both the inhibition of mTOR signaling by rapamycin or silencing Mtor expression in the NAcc with a shRNA during induction ameliorated methamphetamine-induced locomotor behavioral sensitization measured in the last induction session (Huang et al. 2018). Interestingly, both cocaine (Xu et al. 2009) and methamphetamine (Xu et al. 2011) locomotor sensitization are accompanied by an increase in GSK3 activity in the NAcc core, but not in the shell. Moreover, GSK3 systemic inhibition

during the acquisition phase prevented the development of cocaine-induced locomotor sensitization (Miller et al. 2009; Xu et al. 2009) an effect also achieved by intra-NAcc core (but not shell) GSK3 inhibition (Xu et al. 2009). A similar result was observed with methamphetamine (Xu et al. 2011). With regard to the role of dopamine and its relationships with intracellular signaling molecules here discussed, previous studies showed that behavioral sensitization to cocaine (Cadoni et al. 2000), amphetamine (Robinson et al. 1988; Paulson and Robinson 1995; Cadoni et al. 2000), morphine (Cadoni and Di Chiara 1999) or nicotine (Cadoni and Di Chiara 2000) is accompanied by an increase in dopamine responsiveness in the NAcc core and also that Akt/Gsk3 signaling is bidirectionally linked to dopaminergic transmission and related behaviors (Beaulieu et al. 2005; Beaulieu et al. 2004; Beaulieu et al. 2007).

Sensitization to amphetamine-induced stereotypic behaviors was reduced by the administration of a GSK3 inhibitor concomitantly with amphetamine during the acquisition phase (Enman and Unterwald 2012). This effect could be related to the inactivation of Akt and the subsequent activation of GSK3 observed in the striatum of mice after increased dopaminergic neurotransmission arising from the administration of amphetamine (Beaulieu et al. 2004). We are not aware of any study assessing the effects of rapamycin on amphetamine sensitization but, interestingly, the inhibition of mTOR or p70S6K abolished the reduction of amphetamine psychomotor sensitization that is seen after intra-NAcc shell cannabidiol administration (Renard et al. 2016).

Similarly to the situation observed with psychostimulants, systemic administration of rapamycin 3 hours before the challenge test also inhibited the expression of alcohol-induced locomotor sensitization in mice (Neasta et al. 2010).

With regard to nicotine, rapamycin administered either systemically or in the basolateral amygdala (BLA), but not in the central amygdala (CeA), before daily nicotine administration or prior to the test after withdrawal prevented the development and expression of nicotine-induced locomotor sensitization, respectively (Gao et al. 2014).

Interestingly, rats with previous subchronic treatment with i.p. methamphetamine are sensitized to methamphetamine-CPP, but this is prevented when the treatment is accompanied by intra-NAcc rapamycin infusions (Narita et al. 2005). On the other hand, chronic morphine elicits tolerance instead of sensitization to the rewarding properties of morphine itself, as measured by CPP (Shippenberg et al. 1988). This is mediated by a decrease in Insulin Receptor Substrate-2 (IRS2)/Akt and mTORC2 signaling in the VTA, which results in an decrease in dopaminergic VTA neurons cell size and dopamine release to the NAcc (although there is an

increased excitability) (Russo et al. 2007; Mazei-Robison et al. 2011). Consistent with these changes, Mtor deletion in the VTA leads to decreased dopamine release and reuptake in the NAcc (Liu et al. 2018). Surprisingly, mTORC1 activity is increased by chronic morphine, but this modulation is not related to the effects of morphine in VTA dopaminergic neurons (Mazei-Robison et al. 2011). This decrease in soma size has been seen either after passive morphine administration or heroin self-administration in rodents (Russo et al. 2007) and also in post-mortem brain samples from human heroin users (Mazei-Robison et al. 2011). Interestingly, there is no evidence of this effect after cocaine, ethanol, or nicotine self-administration (Mazei-Robison et al. 2014), confirming the heterogeneity of mechanisms of tolerance development to different drugs. The development of tolerance to the analgesic effects of morphine also seems to be mediated by mTOR, since it is blocked by spinal rapamycin administration in rats (Xu et al. 2014).

Table 2: Summary of the studies of the involvement of mTOR system in the acquisition, expression, reconsolidation and reinstatement of locomotor sensitization. C, pairing session; wd, withdrawal day; T, test session (the day after the last wd day); min, minutes; h, hours; d, days; wk, weeks; meth, methamphetamine; Mmu, Mus musculus; Rno, Rattus norvegicus; PND, postnatal day. Rapamycin, inhibitor of mTOR; RapaLink-1, inhibitor of mTORC1; 10-DEBC, inhibitor of Akt; SC79, activator of Akt; SB216763, inhibitor of GSK3; LY294002 and Wortmannin, inhibitors of PI3K; PD98059, PD334581 and U0126, inhibitors of MEK.

	Manipulation/Treatment			Index/Effect			
Drug and Protocol	**What**	**Where**	**When**	**When**	**Effect**	**Model**	**Reference**
Expression of locomotor sensitization during acquisition							
Cocaine 15mg/kg i.p.; C:1/dx5d	LY294002 100μg (iPI3K)	i.c.v.	C1-5 -20min	C1-5	↓	*Rno* ♂	Izzo 2002
Cocaine 15mg/kg i.p.; C:1/2dx8d	Rapamycin 10mg/kg	i.p.	C1-4 -1h	C1-4	~	*Mmu* ♂ PND42-56	Bailey 2012
Cocaine 10mg/kg i.p.; C:1/dx14d	LiCl 100mg/kg (iGSK3)	i.p.	C1-14 -30min	C1-14	↓	*Rno* ♂ 220-240g	Xu 2009
	SB216763 1ng/side	NAc shell			~		
	SB216763 0.1ng/side	NAc core			~		
	SB216763 1ng/side				↓		
Methamphetamine 1mg/kg i.p.; C:1/dx14d	LiCl 100mg/kg (iGSK3)	i.p.	C1-14 -30min	C1-15	↓	*Rno* ♂ 220-240g	Xu 2011
Methamphetamine 1mg/kg i.p.; C:1/dx14d	SB216763 1ng/side	NAc shell	C1-14 -30min	C1-16	~		Xu 2011
Methamphetamine 1mg/kg i.p.; C:1/dx14d	SB216763 1ng/side	NAc core	C1-14 -30min	C1-17	↓		Xu 2011
Nicotine 0.35mg/kg s.c.; C:1/dx5d	Rapamycin 50μg/side	BLA	C1-5 -30min	C1-18	↓	*Rno* ♂ 220-250g	Gao 2014
	Rapamycin 50μg/side	CeA	C1-5 -30min	C1-19	~		
	Rapamycin 1mg/kg	i.p.	C1-5 -45min	C1-20	~		
	Rapamycin 3mg/kg				~		
	Rapamycin 10mg/kg				↓		
Alcohol 2g/kg i.p.; C:1/dx11d	Rapamycin 10mg/kg	i.p.	C11 -3h	C1-21	↓	*Mmu* ♂ PND63-105	Neasta 2010
Methamphetamine 2mg/kg i.p.; C:1/dx8d	Rapamycin 3mg/kg	i.p.	C1-8 -30min	C1-22	~	*Mmu* ♂ PND42-56	Huang 2018
	Rapamycin 6mg/kg				↓		
	LV-mTOR-shRNA7425	NAc shell	C1 -4d	C1-23	↓		
Induction of locomotor sensitization							
Cocaine 15mg/kg i.p.; C:1/dx5d +10wd	LY294002 100μg (iPI3K)	i.c.v.	C1-5 -20min	T	~	Rno ♂	Izzo 2002
Cocaine 15mg/kg i.p.; C:1/dx5d +13wd	Rapamycin 0.1mg/kg	i.p.	C1-5 -5min	T	~	*Rno* ♀ 225-250g	Wu 2011
	Rapamycin 1mg/kg				↓		
	Rapamycin 2.5mg/kg				↓		
	Rapamycin 5mg/kg				↓		
Cocaine 20mg/kg i.p.; C:1/dx5d +7wd	SB216763 2.5mg/kg	i.p.	C1-5 -5min	T	↓	*Mmu* ♂ PND56	Miller 2009
Cocaine 15mg/kg i.p.; C:1/dx5d +10wd	LY294002 100μg (iPI3K)	i.c.v.	T -20min	T+1wk	~	Rno ♂	Izzo 2002
Cocaine 15mg/kg i.p.; C:1/dx3d +14wd	PD98059 1μM	VTA	C1-3, -20min	T +2wk	↓	Rno ♀ 250-300g	Pierce 1999
	PD98059 10μM						
Expression of locomotor sensitization after withdrawal							
Cocaine 15mg/kg i.p.; C:1/dx5d +13wd	Rapamycin 5mg/kg	i.p.	wd10-13	T	↓	*Rno* ♀ 225-250g	Wu 2011
Cocaine 15mg/kg i.p.; C:1/2dx8d +21wd	Rapamycin 10mg/kg	i.p.	T -1h	T	↓	*Mmu* ♂ PND42-56	Bailey 2012
Cocaine 15mg/kg i.p.; C:1/dx5d +10wd	LY294002 100μg (iPI3K)	i.c.v.	T -20min	T	↓	Rno ♂	Izzo 2002
Cocaine 10mg/kg i.p.; C:1/dx14d +5wd	LiCl 100mg/kg	i.p.	T -30min	T	↓	*Rno* ♂ 220-240g	Xu 2009
	SB216763 0.1ng/side	NAc core			~		
	SB216763 1ng/side				↓		

1.4.5.2. Conditioned place preference

The study of CPP not only sheds light about the involvement of the mTOR network in the rewarding properties of drugs but also about the role of this signaling cascade in the development of context-drug associations, which are a crucial part of addiction development and relapse. See Table 2 for a summary of the relevant results of the studies reviewed below.

Table 3: Summary of the studies of the involvement of mTOR system in acquisition, expression, reconsolidation and reinstatement of conditioned place preference. C, conditioning session; T, test session (the day after the last C day); E, extinction session; R, reinstatement session; min, minutes; h, hours; d, days; wk, weeks; amph, amphetamine; meth, methamphetamine; Mmu, Mus musculus; Rno, Rattus norvegicus; PND, postnatal day. Rapamycin, inhibitor of mTOR; RapaLink-1, inhibitor of mTORC1; 10-DEBC, inhibitor of Akt; SC79, activator of Akt; SB216763, inhibitor of GSK3; LY294002 and Wortmannin, inhibitors of PI3K; PD98059, PD334581 and U0126, inhibitors of MEK.

Manipulation/Treatment				**Index/Effect**			
Drug and protocol	**What**	**Where**	**When**	**When**	**Effect**	**Model**	**Reference**
ACQUISITION (treatment during conditioning sessions and test the day after)							
Alcohol 1.8g/kg i.p.; C:1/2dx6d	RapaLink-1, 1.5mg/kg (iMTORC1)	i.p.	C1 -3h	T	~	*Mmu* ♂ PND56-63	Morisot 2018
Amphetamine 1.5mg/kg i.p.; C:1/2dx8d	SB216763 1mg/kg (iGSK3)	i.p.	C1-4 -1h	T	~	*Rno* ♂ 225-250g	Wickens 2016
	SB216763 2mg/kg (iGSK3)				↓		
	SB216763 2.5mg/kg (iGSK3)				↓		
Amphetamine 20ug/side NAc; C:1/2dx8d	SB216763 0.03μg/side (iGSK3)	NAcc			↓		
	SB216763 0.3μg/side (iGSK3)						
	SB216763 3μg/side (iGSK3)						
	SB216763 5μg/side (iGSK3)						
Amphetamine 20ug/side NAc; C:1/2dx8d	PD98059 1μg/side (iMEK)	NAcc	C1-4 -10min	T	~	*Rno* ♂ 200-250g	Gerdjikov 2004
	PD98059 1.7μg/side (iMEK)				~		
	PD98059 2.5μg/side (iMEK)				↓		
			C1-4 0min		↓		
Cocaine 10mg/kg i.p.; C:1/dx4d	SB216763 2.5mg/kg (iGSK3)	i.p.	C1-4 -5min	T	↓	*Mmu* ♂ PND56	Miller 2014
		NAcc			↓		
Cocaine 15mg/kg i.p.; C:1/2dx8d	Rapamycin 10mg/kg	i.p.	C1-4 -1h	T	~	*Mmu* ♂ PND42-56	Bailey 2012
Cocaine 15mg/kg i.p.; C:1/2dx8d	U0126 0.1μg/side (iMEK)	VTA	C1-4 -20min	T	↓	*Rno* ♂ 300-350g	Pan 2011
Cocaine 15mg/kg i.p.; C:2/dx2d	mTOR gene deletion	VTA	C1 -2wk	T	↓	*Mmu* ♂ PND56-70	Liu 2018
Methamphetamine 2mg/kg i.p.; C:1/dx5d	Rapamycin 0.025pmol	NAcc	C1-5 -5min	T	~	*Rno* ♂ 250g	Narita 2005
Morphine 7.5mg/kg s.c.; C:1/dx5d	Rapamycin 15ng/side	CA3	C1-5 -30min	T	↓	*Rno* ♂ 200-250g	Cui 2010
	LY294002 5mM/side (iPI3K)				↓		
		VTA			~		
		NAcc			~		
		CA1			~		
Morphine 10mg/kg i.p.; C:1/2dx8d	SC79 6 μg/side (Akt activator)	vHPC-mPFC (contralateral)	C1-4 -30min	T	~	*Mmu* ♂ PND56-63	Wang 2018
	10-DEBC 0.5μg/side (iAkt)				~		
	PD334581 1 μg/side (iMEK)				↓		

EXPRESSION (treatment previus to the test session)							
Alcohol 1.8g/kg i.p.; C:1/2dx6d	Rapamycin 10mg/kg	i.p.	T -3h	T	↓	*Mmu* ♂ PND63-105	Neasta 2010
Alcohol 1.8g/kg i.p.; C:1/2dx6d	RapaLink-1, 0.75mg/kg	i.p.	T -3h	T	~	*Mmu* ♂ PND56-63	Morisot 2018
	RapaLink-1, 1.5mg/kg		T+1d -3h	T+1d	↓		
Amphetamine 1.5mg/kg i.p.; C:1/2dx8d	SB216763 1mg/kg (iGSK3)	i.p.	T -1h	T	~	*Rno* ♂ 225-250g	Wickens 2016
	SB216763 2mg/kg (iGSK3)				~		
	SB216763 2.5mg/kg (iGSK3)				↓		
Amphetamine 20ug/side NAc; C:1/2dx8d	SB216763 0.03µg/side (iGSK3)	NAcc			~		
	SB216763 0.3µg/side (iGSK3)				↓		
	SB216763 3µg/side (iGSK3)				↓		
	SB216763 5µg/side (iGSK3)				↓		
Cocaine 15mg/kg i.p.; C:1/2dx8d	Rapamycin 10mg/kg	i.p.	T -1h	T	↓	*Mmu* ♂ PND42-56	Bailey 2012
Cocaine 15mg/kg i.p.; C:1/2dx8d	U0126 0.1µg/side (iMEK)	VTA	T -20min	T	~	*Rno* ♂ 300-350g	Pan 2011
Cocaine 15mg/kg i.p.; C:1/2dx8d	Wortmannin 50µ (iPI3K)	vmPFC	T -5min	T	↑CPA	*Mmu* ♂ PND56	Szumlinski 2018
RECONSOLIDATION (treatment in the chamber one day after the test)							
Alcohol 0.5mg/kg s.c.; C:1/2dx8d	Rapamycin 10mg/kg	i.p.	T+1d	T+2d	↓	*Rno* ♂ 220-250g	Lin 2014
				T+15d	↓		
Alcohol 1.8g/kg i.p.; C:1/2dx6d	RapaLink-1, 0.75mg/kg	i.p.	T -3h	T+1d	~	*Mmu* ♂ PND56-63	Morisot 2018
Cocaine 10mg/kg i.p.; C:1/2dx8d	Rapamycin 10mg/kg	i.p.	T+1d	T+2d	↓	*Rno* ♂ 220-250g	Lin 2014
				T+15d	↓		
Cocaine 10mg/kg i.p.; C:1/2dx8d	SB216763 1mg/kg (iGSK3)	i.p.	T+1d	T+2d	~	*Mmu* ♂ PND56	Shi 2014
	SB216763 2.5mg/kg (iGSK3)				↓		
	SB216763 5mg/kg (iGSK3)				↓		
	SB216763 1mg/kg (iGSK3)			T+9d	~		
	SB216763 2.5mg/kg (iGSK3)				↓		
	SB216763 5mg/kg (iGSK3)				↓		
Cocaine 10mg/kg i.p.; C:1/2dx8d	Rapamycin 10mg/kg	i.p.	T+1d	T+2d	↓	*Rno* ♂ 220-250g	Lin 2014
				T+15d	↓		
Morphine 10mg/kg s.c.; C:1/2dx8d	Rapamycin 1mg/kg	i.p.	T+1d	T+2d	~	*Rno* ♂ 220-250g	Lin 2014
	Rapamycin 10mg/kg				↓		
	Rapamycin 1mg/kg			T+15d	~		
	Rapamycin 10mg/kg				↓		
REINSTATEMENT (retest after a challenge two days after extinction)							
Alcohol 1.8g/kg i.p.; C:1/2dx6d; E:4d	Rapamycin 10mg/kg	i.p.	R -3h	R	↓	*Mmu* ♂ PND56	Ben Hamida 2018
Morphine 10mg/kg i.p.; C:1/2dx8d; E:8d	SC79 6 µg/side (Akt activator)	vHPC-mPFC	C1-4 -30min	R	↓	*Mmu* ♂ PND56-63	Wang 2018
Morphine 10mg/kg i.p.; C:1/2dx8d; E:8d	10-DEBC 0.5µg/side (iAkt)	(contralateral)			~		Wang 2018
Morphine 10mg/kg i.p.; C:1/2dx8d; E:8d	PD334581 1 µg/side (iMEK)				~		Wang 2018
C, conditioning session; T, test session (the day after the last C day); E, extinction session; R, reinstatement session; min, minutes; h, hours; d, days; wk, weeks.							

It has been observed that cocaine CPP is accompanied by mGLUR1-dependent increases in ERK, mTOR, P70S6K, S6, eIF4E and eEF1A phosphorylation and activation in the ventral tegmental area (VTA) (Yu et al. 2013; Pan et al. 2011; Miller and Marshall 2005). Moreover, mTOR deletion in the VTA attenuates cocaine CPP and blocks several synaptic features associated to cocaine CPP such as the increase in the AMPAR/NMDAR ratio, the increase ind both frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) of VTA dopaminergic neurons and the decrease in both frequency and amplitude of spontaneous inhibitory post-synaptic current (sIPSCs) of VTA GABAergic neurons (Liu et al. 2018). Surprisingly, a systemic rapamycin treatment prior to cocaine conditioning sessions does not affect the magnitude of the place-preference exhibited on the test (Bailey et al. 2012). Intra-NAcc rapamycin microinjections before conditioning sessions also failed to prevent methamphetamine CPP development (Narita et al. 2005). On the other hand, systemic rapamycin administered before the preference test does ameliorate cocaine CPP expression (Bailey et al. 2012). Alcohol CPP is also decreased by systemic administration of rapamycin before the test (Neasta et al. 2010) and also by the systemic administration of RapaLink1, an mTORC1 specific inhibitor (Morisot et al. 2018). These data suggest that mTOR signaling is only required for the expression of CPP, but not for its acquisition. On the other hand, bilateral administration of a MEK inhibitor into the VTA during acquisition blocked cocaine CPP but did not hinder its expression when administered before the test (Pan et al. 2011). Similarly, bilateral MEK inhibition in the NAcc during the conditioning sessions blocked amphetamine CPP induction (Gerdjikov et al. 2004).

Interestingly, the administration before the preference test of a PI3K inhibitor into the ventromedial prefrontal cortex (vmPFC) of mice which had previously shown preference for the cocaine-associated chamber not only inhibited CPP expression but even evoked aversion to the cocaine-paired compartment, implicating PI3K activity in the vmPFC in the motivational valence of cocaine-associated cues (Szumlinski et al. 2018).

The administration of a GSK3 inhibitor in rats either systemically or directly into the NAcc was able to block both the acquisition and the expression of amphetamine CPP (although lower doses were required to block the acquisition) (Wickens et al. 2016). Coincidently, cocaine CPP development was also prevented by systemic GSK3 inhibition in mice during the conditioning sessions (Miller et al. 2014).

Morphine CPP was accompanied by an increase in phosphorylation levels of Akt, mTOR, and P70S6K in the hippocampal CA3 field. Moreover, CPP scores significantly correlated with the phosphorylation ratios of these proteins in CA3. A pretreatment

with rapamycin or with the PI3K inhibitor LY294002 microinjected in CA3 completely abolished morphine CPP expression while it remained unchanged by intra-VTA, NAcc or CA1 LY294002 microinjections. Morphine CPP is mediated by PI3K/Akt/mTOR activation in CA3 and this is dependent on μ opioid receptor activation since blockade of this receptor in CA3 prevented both CPP and the increased levels of mTOR related proteins (Cui et al. 2010). Also, after morphine CPP training, pERK1 was increased in vHip and mPFC in the morphine-paired group. Contralateral inhibition of the pERK activator MEK on those areas before the conditioning sessions completely blocked morphine CPP (Wang et al. 2018).

1.4.5.3. Self-administration

Operant cocaine self-administration was not altered by intra-NAcc shell rapamycin administration, although it reduced responding during non-drug available periods (inactive lever presses unaffected), which could be interpreted as a decrease in impulsive drug-seeking responding (James et al. 2014). Cocaine self-administration was also unaffected by intra-NAcc core rapamycin administration (Wang et al. 2010). Rats with diminished expression of Gsk3b in the NAcc shell showed increased cocaine self-administration rates during acquisition and maintenance, and at the higher doses of a dose-response curve (Crofton et al. 2017). After cocaine self-administration (24 hours after the last session), rats had higher levels of total mTOR and phospho-mTOR in the NAcc shell but not in the core, although total p70S6K levels were higher in the NAcc core. Unfortunately, the experimental design did not allow to discern if these results were a consequence of the self-administration protocol or withdrawal (James et al. 2014).

Although there is no clear evidence of the benefits of mTORC1 inhibition in psychostimulant administration under fixed-ratio schedules, mTORC1 may be involved in psychostimulant responding under high effort conditions. Intracerebroventricular rapamycin administered in rats 3 hours before cocaine self-administration under a progressive ratio schedule, lowered the breakpoints as compared to the previous rapamycin-free baselines. In the same study, progressive ratio breakpoints were also reduced by intra-NAcc shell injections (James et al. 2014). Similarly, decreases in lever presses and breakpoints in cocaine self-administration under progressive ratio schedules were observed with systemic rapamycin pretreatment, which increases the therapeutic value of mTOR inhibition (James et al. 2016).

In studies using rodent models of excessive voluntary alcohol consumption comparable to human binge drinking, an increase in the NAcc of phosphorylated levels of PI3K, Akt, GSK3, 4EBP and P70S6K was observed (Cozzoli et al. 2009; Liu et al. 2017;

Neasta et al. 2010; Neasta et al. 2011). Moreover, binge drinking and sustained consumption of alcohol could be decreased by rapamycin administration (systemic or intra-NAcc) prior to the alcohol drinking session (Neasta et al. 2010) and also by the newly developed mTORC1 inhibitor RapaLink-1 (Morisot et al. 2018). A similar effect was achieved through intra-NAcc administration of wortmannine (a PI3K inhibitor) (Neasta et al. 2011; Cozzoli et al. 2009) or triciribine (an Akt inhibitor) (Neasta et al. 2011). Some of the downstream proteins involved in this behavior have been identified among the ones show increased translation by mTORC1 activity. For example, some glutamatergic transductors such as Gria1 (GluA1 AMPA receptor subunit), Grm5 (mGlu5 metabotropic glutamate receptor) and Homer2 are overexpressed in the NAcc of animals with a history of excessive voluntary consumption, and alcohol consumption was attenuated by downregulating Homer2 expression or administrating mGlu5 antagonists in the NAcc (Cozzoli et al. 2012; Neasta et al. 2010; Cozzoli et al. 2009). Collapsin response mediator protein-2 (CRMP-2), a microtubule-binding protein that regulates microtubule assembly (Ip et al. 2014), is also increased in the NAcc after excessive voluntary drinking, which also blocks its phosphorylation by GSK3, leading to an increase in microtubule content. Moreover, excessive alcohol consumption was decreased after inhibiting systemically CRMP-2 or downregulating Crmp2 expression in the NAcc (Liu et al. 2017). Other protein identified is Prosapip1 which is also overexpressed in mice excessively consuming alcohol, leading to changes in dendritic spine morphology and GluA2 lacking AMPA receptors location in NAcc medium spiny neurons (MSNs) (Laguesse et al. 2017b). When the NAcc subdivisions shell and core have been studied separately, the results point out the shell as the area where this modulation of the mTOR network occurs. A single binge session of an alcohol voluntary consumption protocol is sufficient to activate mTOR machinery in dopaminergic receptor D1-expressing cells in NAcc shell resulting in an increase in the levels of GluA1 and Homer and eliciting synaptic plasticity, effects that are absent in the NAcc core. The increase in mTORC1 activity seems to be mediated by the activation of D1 receptors after the alcohol-elicited increase in dopamine release in the NAcc. When mTORC1 was inhibited by rapamycin, alcohol consumption in a subsequent binge drinking session was decreased (Beckley et al. 2016), suggesting an important role of this pathway in the escalation of alcohol consumption. Another study that assessed the activation of mTORC1 and mTORC2 in corticostriatal areas of rats and mice after excessive voluntary ethanol intake, found that increased specific phosphorylations of S6 and Akt only in the NAcc shell, but not in the core, an effect still evident after 24 hours of withdrawal. In addition to the results in the NAcc the authors also found increased activity of both complexes in the orbitofrontal cortex (OFC), but not in the medial prefrontal cortex (mPFC), and also an increase, only in mice, in mTORC2 activity in the dorsomedial striatum (DMS) (but not in the dorsolateral striatum –DLS-) (Laguesse et

al. 2017a). A similar protocol of alcohol consumption in mice elicited increases in Sgk1 and Gsk3 phosphorylation in the DMS, but not in the DLS, supporting the mTORC2-dependent increase in Akt phosphorylation. Moreover, mTORC2 activity in the DMS contributed to the development of alcohol binge drinking (as revealed by manipulations involving the activation or repression of the pathway) in parallel with an increase in length and complexity of the dendritic branches, and in the size and stability of the dendritic spines of DMS MSNs (Laguesse et al. 2018). Interestingly, mTORC2 seems to modulate dopaminergic responses in the dorsal striatum, since neuron-specific Rictor knockout mice show increased dopamine transporter (DAT) expression and activity in this area and a heightened behavioral and physiological (dopamine release) responses to amphetamine (Dadalko et al. 2015).

In alcohol operant self-administration studies, systemic rapamycin decreased alcohol self-administration and seeking in non-reinforced seeking tests (Neasta et al. 2010). Intra-NAcc inhibition of PI3K or Akt had a similar effect on alcohol operant responding (Neasta et al. 2011). Neither systemic rapamycin nor intra-NAcc inhibition of PI3K or Akt affected sucrose operant self-administration, suggestive of specificity in these results (Neasta et al. 2010; Neasta et al. 2011).

The mTOR pathway also has a role in alcohol-induced increase in impulsive behavior which is an endophenotype that predisposes to addiction (Belin et al. 2008; Dalley et al. 2007; Dalley and Ersche 2019). By using label-free proteomics, Starski and colleagues identified significant protein expression changes in the mTOR pathway in the anterior cingulate cortex between control and ethanol-induced impulsive mice (Starski et al. 2019). It still remains to be determined if trait impulsivity also shows a similar dependency of mTOR dynamics, which could be useful for the development of pharmacotherapies of impulsive disorders.

Relating to opiate self-administration, we are not aware of any studies that have examined the effects of mTOR manipulations on morphine, heroin, or synthetic opioids self-administration. However, we have recently shown that morphine self-administration was associated to enduring increments in the gene expression of Raptor and Eif4bp2 in the amygdala of male Lewis rats and these effects were still evident even after extinction training (Ucha et al. 2019). We have also observed that the gene expression of several elements of the mTOR network such as Akt2, GSK3a, insulin like growth factor receptors 1 and 2, or Rictor is increased in the basal complex of the amygdala (basolateral+basomedial nuclei) after extended access (6 hours per day) to heroin self-administration in male Lewis rats (unpublished observations).

In addition to the animal models discussed here, there is evidence from humans of the implication of mTOR in alcohol addiction. There are genetic variants of MTOR,

EIF4E and EEF1A which predicted the number of drinking days per month in two independent samples. Moreover, a CpG island of the EEF2 gene was found to be hypermethylated in the heaviest drinkers (Meyers et al. 2015).

1.4.5.4. Reinstatement

Relapse into drug use is the major problem in recovering addicts. There are several adaptation of the CPP and self-administration paradigms that model a human relapse. One of such models is the increase of cue-reactivity (revealed by conditioned cue-contingent lever presses under extinction conditions) after increasing periods of forced withdrawal, the so-called ‘incubation of craving’ paradigms (Grimm et al. 2001; Pickens et al. 2011).

Seemingly, there are different mechanisms in the NAcc core regulating incubation and expression of cocaine seeking. Rats with incubated cocaine-seeking show an increase in S6 phosphorylation, independently of the rats having or not a seeking test, but only the rats that performed the seeking tests shown an increase in phosphorylated ERK and 4E-BP and a decrease in phosphorylated eEF2 and eIF2α, which accounts for an increase in protein translation. Subsequently, blocking either mTORC1 with rapamycin or eIF2α dephosphorylation with a selective phosphatase inhibitor in the NAcc core before the seeking tests reduces cue-induced cocaine-seeking (Werner et al. 2018). Interestingly, eIF2α seems to be also implicated in the development of cocaine-induced CPP (Huang et al. 2016) and LTP in DA neurons in the VTA (Placzek et al. 2016b; Huang et al. 2016),and in reward-dependent striatal activity in human tobacco smokers (Placzek et al. 2016a). Rapamycin prevents the elevated transmission mediated by GluA2-lacking Ca2+-permeable AMPA receptors found in the NAcc core of rats after incubation of cocaine craving (Scheyer et al. 2014), which is known to mediate the expression of cocaine craving (Conrad et al. 2008; Loweth et al. 2014). Such an effect could also account for the decrease in reinstatement induced by intraNAcc rapamycin or eIF2α phosphatase inhibitors, coincidentally with the diminished levels of GluA1 in the NAcc after rapamycin administration (James et al. 2014; James et al. 2016), which could explain the effects on reinstatement. Rapamycin administered into the NAcc also attenuates cocaine-associated cue-induced reinstatement in rats that undergo extinction training, an effect found both with rats that have intra-NAcc rapamycin administered before extinction (James et al. 2014) or when rapamycin is administered in the NAcc core (but not the shell) before the seeking test (Wang et al. 2010). The exposure to cocaine-related cues after extinction evokes an increase in phosphorylated P70S6K and S6 in the NAcc core, but not in the shell (Wang et al. 2010). Interestingly, rats selected for being vulnerable for cue-induced relapse to cocaine-seeking had lower expression of several genes related to the

network, such as Mtor, Pik3ca, and Prkcb1 in the NAcc and Pik3ca and Prkcb1 in the striatum (Brown et al. 2011). However, while cocaine self-administration in both mice and rats does not affect phosphorylated PI3K levels in the vmPFC, they are increased after 21 days of withdrawal. In spite of this, rats that undergo seeking tests after 3 and 30 days of cocaine withdrawal have decreased total Akt levels and increases in the relative expression of p-Akt in the vmPFC. These alterations were absent in rats that were not subjected to seeking tests. Also, there is a positive correlation between PI3K activation in the vmPFC and cue reinforced lever presses. In accordance to these results, an intra- vmPFC (withing the prelimbic cortex) infusion of wortmannin (a PI3K inhibitor) before the test blocks the incubated seeking (Szumlinski et al. 2018). Of note, the protecting effect of rapamycin against reinstatement does not apply to natural reinforcers, since intra-NAcc rapamycin infusions do not attenuate reinstatement for sucrose seeking (Wang et al. 2010).

CPP models have also revealed a role for the mTOR pathway in relapse. Reinstatement of alcohol CPP (by injecting a priming dose of the drug after the extinction of the conditioned preference) is accompanied by an increase in mTORC1 signaling, measured by S6 phosphorylation, in the NAcc shell, but not the NAcc core (unlike cocaine reinstatement) or the striatum. Moreover, S6 phosphorylation levels in the NAcc are proportional to the reinstatement score, and systemic rapamycin administration before the test completely blocks CPP reinstatement, supporting the involvement of mTORC1 in alcohol reinstatement (Ben Hamida et al. 2018). The authors of this study identified CRMP2 as the downstream responsible for alcohol reinstatement in the NAcc shell (Ben Hamida et al. 2018). CRMP2 is a microtubule-binding protein that regulates microtubule assembly (Ip et al. 2014) also involved in excessive voluntary drinking (Liu et al. 2017), which makes it a suitable candidate as a regulator of the synaptic changes behind alcohol addiction. With regard to opiates, reinstatement to morphine CPP elicits a decrease in Akt phosphorylation (in the site phosphorylated by PDK1) in the ventral hippocampus and the mPFC. Moreover, reinstatement is blocked by administering an Akt activator in one of the ventral hippocampus and the contralateral mPFC (Wang et al. 2018).

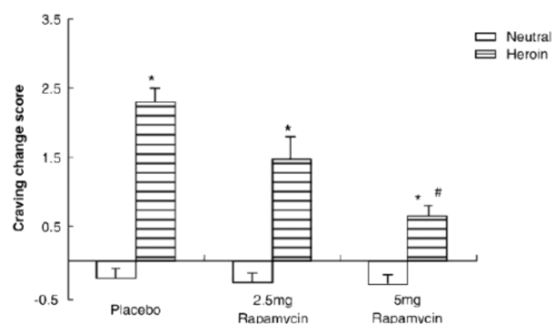


*Figure 13: Effect of rapamycin on cue-induced drug craving in abstinent heroin human addicts. Means change scores for craving in response to neutral and heroin cues. *p<o.o5 compared to neutral image within the same group. # p<0.05 compared to Placebo and 5mg Rapamycin (Shi et al., 2009)*

Finally, only one study that we are aware of has been performed to examine the therapeutic value of rapamycin in the prevention of reinstatement in humans. This study revealed that rapamycin administered orally two hours before

exposing heroin addicts to drug- (heroin) related cues (in a film) reduces cue-induced heroin craving in a dose-dependent manner, leaving anxiety measures unaffected (Figure 13) (Shi et al. 2009).

1.4.5.5. Drug-memory reconsolidation

Another interesting phenomenon regulated by the mTOR network is the reconsolidation of drug-associated memories which can be studied in rats undergoing morphine, cocaine or alcohol CPP training (see Box 1). For example, it has been observed that if rats are re-exposed to the drug-paired chamber 24 hours after the last conditioning session (so that drug-associated memories are reactivated), systemic rapamycin administration immediately after this retrieval session reduces drug-seeking tested by CPP expression (24 hours and 14 days after the retrieval session). This effect was not seen when the retrieval session followed by rapamycin was performed in the vehicle-associated chamber, showing that the effect of rapamycin was specific of the memories retrieved by the cues (Lin et al. 2014). In another study, phosphorylation levels of Akt, GSK3, mTOR, and p70S6K were decreased immediately after the retrieval of cocaine-associated memories in the NAcc and the hippocampus of mice. A similar effect was observed in the PFC for the phosphorylation levels of Akt and GSK3, and the same trend was found in the striatum (Shi et al. 2014). Of note, the effects of cocaine memory reactivation on GSK3 levels mentioned before have recently been shown to be dependent on NMDA receptor activation (Shi et al. 2019). The results regarding memory reactivation and mTOR function may seem conflicting, as memory retrieval, which is prevented by rapamycin (inhibitor of mTORC1) promotes the inhibition of mTORC1 mediators. Moreover, administration of a GSK3 inhibitor after the retrieval session also disrupted memory reconsolidation (Shi et al. 2014). As GSK3 activity elicits mTORC1 inhibition, this result is at odds with rapamycin blockade of memory reconsolidation.

In another study, reactivation of alcohol-related memories in rats induced mTORC1 activation measured by phosphor-S6+ cell count specifically in the CeA and the prelimbic (PrL) and orbitofrontal cortices, but not in infralimbic cortex (IL), NAcc, BLA or dorsal hippocampus. Higher levels of phospho-4EBP, phospho-P70S6K and phospho-S6 were also found in the amygdala, the vmPFC, and the OFC. Rapamycin administered either systemic or directly into the CeA, after the retrieval session disrupted memory reconsolidation of alcohol-related memories, leading to long-lasting suppression of relapse (Barak et al. 2013).

Hypotheses and goals

The general goal of this Thesis has been to increase and improve our understanding of several aspects related to opiate use disorders, from impulsivity, a risk factor for the development of addiction, to different animal models of relapse. We have evaluated the potential relationship of impulsivity and relapse with several elements of the glutamatergic, GABAergic and endocannabinoid systems or the mTOR intracellular signalling cascade.

Our hypotheses and objectives were:

- **Hypothesis 1:** Given that the mOFC and lOFC have opposing roles in the regulation of impulsivity as assessed in the DDT (Mar et al., 2011), we supposed that this dissociation would also be reflected in the glutamatergic and GABAergic dynamics of these territories of the OFC. In addition, we hypothesised that a different pattern of results would be obtained for impulsivity that is captured by the 2-CSRTT. Indeed, because glutamatergic and GABAergic neurotransmission is directly related to the excitation or inhibition status and both are regulated by endocannabinoid neuromodulation we hypothesized that the expression of genes related to glutamatergic, GABAergic or cannabinoid neurotransmission in the lOFC or mOFC could be related to the two varieties of waiting impulsivity that are captured by the DDT or the 2-CSRTT.

    - **Goal 1:** To analyze the relationship between the expression of several genes related to glutamatergic, GABAergic or cannabinoid neurotransmission in the lOFC and mOFC and the impulsivity measured either by the DDT or the 2-CSRTT.

Hypothesis 2: Substance use disorders emerge from neuroadaptations in several areas that govern different aspects of behaviour. These changes are mediated by intracellular signalling networks, however, our knowledge about how these mechanisms are involved in the different aspects of drug reward and drug-induced neuroadaptations that participate in substance use disorders remains very limited (see point 1.4 of this thesis). There are some reports in the literature about how opioids can directly affect the mTOR signalling network (Mazei-Robison et al., 2011; Muller and Unterwald, 2004; Olianas et al., 2011; Polakiewicz et al., 1998; Russo et al., 2007; Wang et al., 2015; Xu et al., 2014). On the basis of this knowledge, we hypothesised that some of the components of this network would be involved altered during self-administration of opioids like morphine or heroin and abstinence (be it during extinction or after drug withdrawal).

- **Goal 2:** To evaluate the effects of morphine self-administration in an extended access schedule, followed by extinction, on the expression and activity of several proteins related to the mTOR signalling network.
- **Goal 3:** To assess the effects of heroin self-administration in an extended access schedule, followed by drug withdrawal, on the expression of several proteins related to the mTOR signalling network.

To test these hypotheses, we ran three experiments with different goals:

Experiment I: We screened Wistar rats in the DDT and the 2-CSRTT tasks, that are supposed to capture two different varieties of waiting impulsivity. We then studied the expression of genes related to glutamatergic, GABAergic or cannabinoid neurotransmission in the lOFC or mOFC of these rats by RT-qPCR and tested their correlation with the parameters obtained in these tasks that are supposed to capture impulsivity better. We assessed the expression of two subunits of the NMDA glutamatergic receptor (R1 and 2A), AMPA receptor (GluA1 and GluA2), $GABA_A$ receptor (alpha 1, alpha 2, delta, and gamma 2) an of elements of the endocannabinoid system (the CB1 receptor, the anandamide synthesis enzyme NAPE-PLD, the anandamide-degrading enzyme FAAH, the 2-arachidonoyl glycerol (2-AG) synthesis enzyme diacylglycerol lipase and the 2-AG degrading enzyme monoacylglycerol lipase).

Experiment 2: Lewis rats self-administered either morphine or saline in an extended access (12 hours) schedule. After that, half of them were sacrificed and the other half underwent an extinction protocol. Then, we studied the effects of these manipulations on the expression and/or activity of several proteins related to the mTOR network. For this purpose, we chose three brain areas known for their involvement in opioid reinforcement and extinction learning: the amygdala, the NAcc, and the prefrontal cortex (PFC). The expression of several mediators of the mTOR pathway was analysed using RT-qPCR. We chose three genes coding membrane receptors related to the pathway (*Igf1r*, *Igf2r* and Insr), seven genes coding upstream intracellular second messengers (*Akt1*, *Akt2*, *Gsk3a*, *Gsk3b*, *Pdk1* and *Pi3ca*), three components of the mTOR complexes (*Mtor*, *Rptor* and *Rictor*) and seven downstream mediators and effectors of the pathway (*Eef1a1*, *Eif4e*, *Rps6kb1*, *Rps6*, *Sgk1* and *Eif4ebp2*). We have also assessed the activation levels of specific proteins encoded by these genes in western blots with phosphospecific antibodies directed to phosphorylation sites required for their

activation by kinases of the pathway. The phosphoproteins assessed were Akt (Ser437), Gsk3α/β (Ser21/9), mTOR (Ser2448), PDK1 (Ser241) and p70 S6 Kinase (Thr389).

Experiment 3: Lewis rats self-administered either heroin or saline in an extended access schedule (6 hours). Half of them were sacrificed 24 hours later, and the other half underwent forced abstinence for 30 days. We then studied the effects of these manipulations on the expression of several proteins related to the mTOR network. In this experiment we focused only in the BLA because it was the area in which we obtained the most interesting results in experiment 2 (Ucha et al., 2019a), and because it is an area with a key role in the formation of drug-related memories and the encoding of their emotional value (for a review see Luo et al., 2013). Moreover, it is also involved in the restatement of heroin-seeking behaviour (Fuchs and See, 2002). Using RT-qPCR, we analyzed the expression in this area of three genes of receptors related to the mTOR network (*Igf1r*, *Igf2r*, and *Insr*), seven genes encoding upstream second messengers of the network (*Akt1*, *Akt2*, *Gsk3*, *Gsk3b*, *Pdk1* and *Pi3ca*), three encoding components mTOR complexes (*mTOR*, *Rptor* and *Rictor*) and three genes encoding downstream mediators and effectors (*Rps6kb1*, *Rps6* and *Eif4ebp2*).

Chapter 2

Functional dissociation of the orbitofrontal cortices regarding waiting impulsivity

Ucha et al., 2019b

2.1. Materials and Methods

2.1.1. Animals

Adult male Wistar rats (n=42, 18 per experiment) (Charles River Laboratories) were housed in groups of 3 in a controlled facility with a temperature of 22±2ºC and relative humidity of 50%±10 on an inverted 12h/12h light/dark cycle (lights on at 8:00 pm). The rats weighed around 300 g at the beginning of the experiments and were kept at around 90-95% of their original weight by restricting their access to food (standard commercial rodent diet A04/A03: Panlab). They had *ad libitum* access to water through all the duration of the experiments. All the animals were maintained and handled according to European Union guidelines for the care of laboratory animals (EU Directive 2010/63/EU governing animal experimentation).

2.1.2. Apparatus

The behavioural tests were performed using six operant conditioning chambers (l=300 mm; w=245 mm; h=328 mm) (Med Associates). The front part of each box was equipped with two levers 14 centimetres apart and a pellet dispenser with a head entry detector between them. There were also light cues above each lever, a house light close to the top of the boxes and a white noise generator. The chambers were controlled using the software MedPC by a computer connected to a compatible interface (Med Associates).

2.1.3. Behavioural Tasks

2.1.3.1. Acquisition of lever press response

All the rats received instrumental training sessions with food pellets (grain-based rodent tablet, Testdiet™) and a light cue indicating the active lever on a fixed ratio 1 schedule. The sessions lasted 30 minutes and continued daily until the animals developed an acceptable lever press behaviour (at least 30 lever presses in one session), and then the same training was performed with the other lever. The order of the levers was counterbalanced across the conditioning chambers, and each animal had an assigned chamber from the beginning of the experiment. Once the animals reached the criterion for both levers, they were trained with both active levers simultaneously (both cue lights on/both levers reward) until the Left/Right lever ratio was 1:1 ±10%.

2.1.3.2. Behavioural measurements of impulsivity

2.1.3.2.1. Delay-discounting task

For the study of "impulsive choice", we used an adaptation of the protocol of the DDT described by Mar and Robbins, 2007 (see Fig. 14). Each session lasted 100 minutes and consisted of five blocks of 12 trials each. Trials are presented every 100

seconds (i.e., 60 trials in 100 minutes). One of the levers (the "immediate lever") initiated the delivery of one food pellet when pressed while the other (the "delayed lever") delivered four of them. The immediate and delayed levers were in the same location (left or right) for each animal, but their position was counterbalanced between animals. The delay between lever press and the delivery of the reward was always 0 seconds for the immediate lever, whereas the delay associated to the delayed lever was increased across blocks in order to assess the tolerance to delay of the rats. The first two trials of each block were forced (i.e. only one lever was active and its corresponding cue light was illuminated). During the rest of the trials both levers were available, a fact that was signalled by the illuminated cues lights above each lever. Once a lever was pressed within the 10 seconds interval given, the cue lights were turned off, and an inter-trial interval commenced. If the rat failed to respond during the 10 seconds, all lights were turned off, punishing the omitted response. During the first training sessions, both levers delivered a reward immediately, and these sessions continued until the rats showed a clear preference for the lever that delivered the large reward (>90% choice). Once the criterion was met, the rats started the test sessions in which the delay of delivery for the delayed lever was increased with every block change (0 seconds, 5 seconds, 10 seconds, 20 seconds and 40 seconds respectively). At the end of each block, a tone cue was presented to mark the beginning of the next block. The choice ratio for each block was calculated by dividing the number of delayed responses in all the free-choice trials of the block (a maximum of 10 free-choice trials per block) by the number of free-choice trials completed. We used the average of the choice ratio during three consecutive blocks as a reliable estimate of choice behaviour.

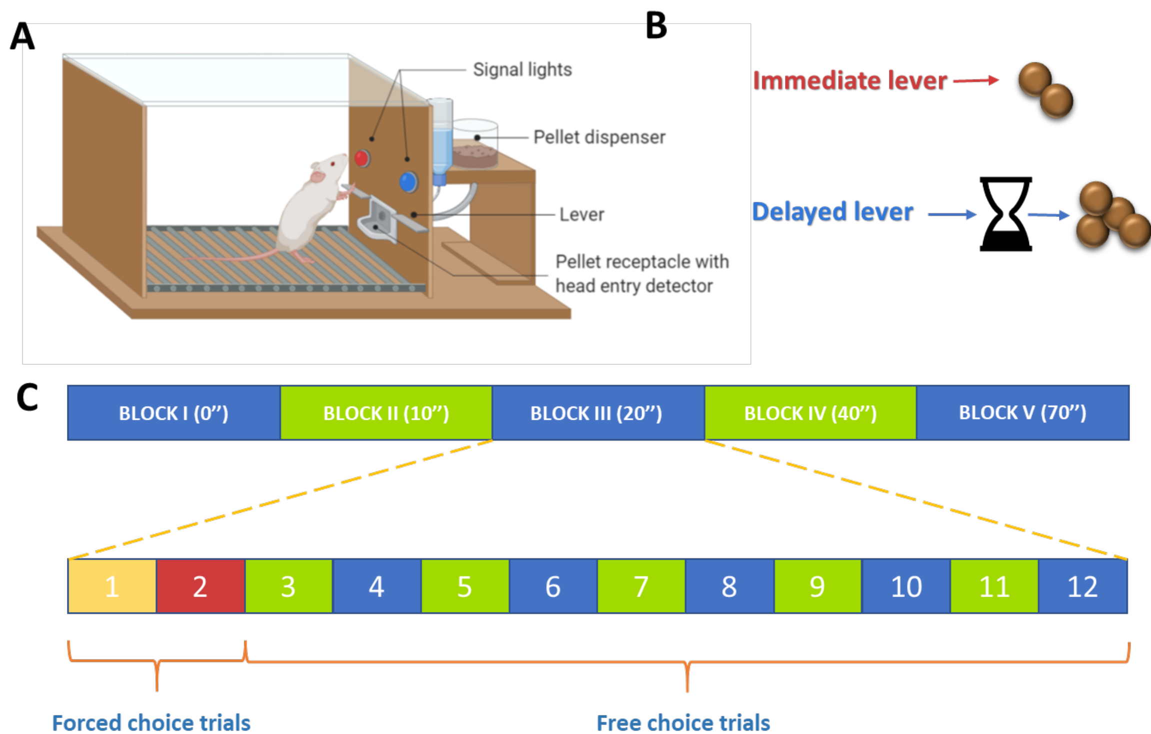


Figure 14: A) Setup of the conditioning chamber used for this procedure. B) During the free-choice trials, responding on the immediate lever results in immediate delivery of one palatable food pellet, meanwhile responding on the delayed lever is rewarded with four pellets, but delayed by a predetermined amount of time. C) Outline of the sessions. Each session consisted of five blocks with increasing delay times for the delayed lever. Each block started with two forced trials followed by ten free-choice trials

The sessions were repeated daily until the rats achieved a stable delay-discounting performance. Due to the variability of discounting curves between rats, the criterion for stability was defined by the average behaviour of all the rats. We performed a two-way repeated-measures analysis of variance (ANOVA) with the average choice ratios during two contiguous 3-sessions blocks as the BLOCK dependent variable and 3-SESSIONS and DELAY as within-subject factors. Stability was met when no significant effect of the 3 SESSION BLOCK was found but a significant effect of DELAY was observed. This was achieved after twenty sessions of delay-discounting training.

Waiting impulsivity was operationalised here by the k parameter, calculated by fitting the choice ratio of the last three sessions block to a nonlinear exponential function ($CR=e^{-k(DELAY)}$). The *k* parameter determines the rate of decay of the exponential function, i.e. the rate at which the lever choice changes from delayed to immediate across delays. Consequently, larger k values indicate a faster rate of lever choice change and more impulsive behaviour (Odum, 2011). There are other methods to compare the behaviour of delay-discounting curves across groups or subjects, like the normalised area under the curve (AUC) (Myerson et al., 2001) or the AUC without normalisation (Magnard et al., 2018). Similarly to the k parameter, these two metrics

provide an index that is comparable between studies. In addition to computing the k parameter, we have also extracted both AUCs measures and tried to cluster the rats using the two indices. The main correlation of this study was preserved using both AUCs. However, the groups resulting from the clustering process had very different sample sizes and were not considered in this study (see Supplementary Information).

2.1.3.2.2. Two choice serial reaction time task

The two choice serial reaction time task (2-CSRTT) used here is an adaptation of the popular five-choice serial reaction time task (Bari et al., 2008). The 2-CSRTT has been shown to be sensitive to an amphetamine challenge which increased premature responding in the task while leaving other parameters unaffected (Van Gaalen et al., 2009). This task was carried out in the same conditioning boxes described for the DDT (Figure 15A). The task started once the nose poke detector sensed an entry in the pellet dispenser and followed the sequence shown in figure 15B.

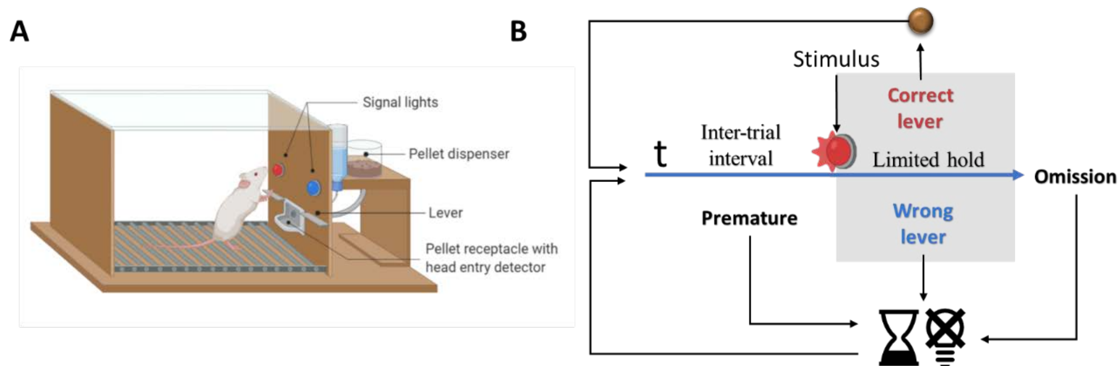


Figure 15: A) Setup of the conditioning chamber used for this procedure. B) The trial sequence of the 2-choice serial reaction time task (2-CSRTT): The task was initiated when a head-entry was detected. Then, one of the stimulus lights was turned on for a variable period. If the lever under the light was pressed during the limited hold period (CORRECT response), a pellet was delivered and, after an inter-trial interval (ITI), the next trial started. If the rat pressed the wrong lever (ERROR response), pressed a lever before any stimulus (PREMATURE response), or did not press any lever at all (OMISSION response), then the house light was turned off, and rewards were not available during 5 seconds as a punishment. The sessions finished after 100 trials or 30 minutes, whichever came first. Once a rat completed one session with more than 75% of correct responses and less than 20% of omissions, the next phase of the experiment started.

The experiment consisted of 12 training phases and a test phase. As the phases progressed, the stimulus duration and response interval time were shortened, while the ITI was extended (as detailed in the excellent description of the protocol by Bari et al., (2008), and summarized here in table 4). In the test phase, the ITI was drastically increased to 9 seconds to increase the number of premature responses and unmask the latent impulsivity trait. We used this variable (number of premature responses during the test phase) as a measure of the motor component of waiting impulsivity of each rat.

Table 4: Overview of the stages involved in the training and testing on the 2-Choice Serial Reaction Time Task (2-CSRTT) (Bari et al., 2008).

Training stage	Stimulus duration (s)	Inter-trial interval (s)	Limited hold (s)	Criterion to move to the next stage
1	30	2	30	≥ 30 Correct trials
2	20	2	20	≥ 30 Correct trials
3	10	5	10	≥ 50 Correct trials
4	5	5	5	≥ 50 Correct trials > 80% Accuracy
5	2.5	5	5	≥ 50 Correct trials > 80% Accuracy < 20% Omissions
6	1.25	5	5	≥ 50 Correct trials > 80% Accuracy < 20% Omissions
7	1	5	5	≥ 50 Correct trials > 80% Accuracy < 20% Omissions
8	0.9	5	5	≥ 50 Correct trials > 80% Accuracy < 20% Omissions
9	0.8	5	5	≥ 50 Correct trials > 80% Accuracy < 20% Omissions
10	0.7	5	5	≥ 50 Correct trials > 80% Accuracy < 20% Omissions
11	0.6	5	5	≥ 50 Correct trials > 80% Accuracy < 20% Omissions
12	0.5	5	5	≥ 50 Correct trials > 75% Accuracy < 20% Omissions
Test	0.5	9	5	

2.1.4. Sample processing

After the behavioural assessments, the animals of both experiments were left *ad libitum* in their home cages for one week, to prevent any effect of the behavioural tests on gene expression. Then, they were mildly anaesthetised with isoflurane and euthanised by decapitation. Using tools and surfaces previously treated with RNAseZap (Ambion) to prevent RNA degradation, the brain was extracted and the mOFC and lOFC were dissected out of 1 mm slices obtained by using a brain matrix and the adequate equipment. The dissected areas are depicted in Figure 16. The samples were then snap-frozen in dry ice and stored at -70°C for further processing. 5 brains of the delay-discounting experiment were lost due to a faulty freezer.

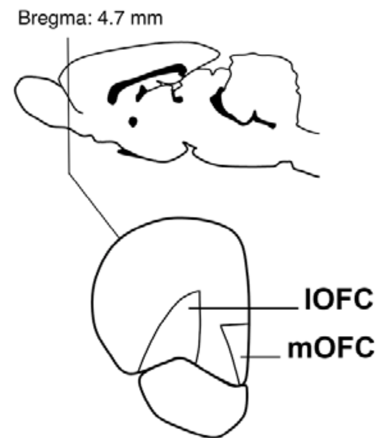


Figure 16: *A cartoon depicting the approximate Bregma level at which dissections were made. The medial and lateral divisions of the orbitofrontal cortex were dissected out on ice with the help of the Paxinos and Watson atlas.*

2.1.5. RT-qPCR

RNA was isolated using the commercial kit RNeasy Lipid Tissue Mini Kit (Qiagen). Samples were retrotranscribed using a commercial kit (Biorad iScript™ cDNA Synthesis Kit). PCR assays were performed on a real-time PCR detection system (CFX9600, Bio-Rad) with an SSO Advanced SYBR mix (Bio-Rad) using the primers indicated in Table 5. We assessed the expression of subunits of the NMDA glutamatergic receptor (R1 and 2A), AMPA receptor (GluA1 and GluA2), $GABA_A$ receptor (alpha 1, alpha 2, delta, and gamma 2) an of elements of the endocannabinoid system (the CB_1 receptor, the anandamide synthesis enzyme NAPE-PLD, the anandamide-degrading enzyme FAAH, 2-arachidonoyl glycerol (2-AG) synthesis enzyme diacylglycerol lipase and the 2-AG degrading enzyme monoacylglycerol lipase). The relative expression of the target genes was calculated according to Pfaffl, 2001, using *Gapdh* as a reference gene and the reaction efficiencies were obtained using LinRegPCR software (Ruijter et al., 2009).

Table 5: Primer sets used for RT-qPCR amplification

Gene	Description	Forward primer	Reverse primer
Grin1	glutamate ionotropic receptor NMDA type subunit 1	AACCTGCAGAACCGCAAG	GCTTGATGAGCAGGTCTATG C
Grin2a	glutamate ionotropic receptor NMDA type subunit 2A	TGTGAAGAAATGCTGCAAGG	GAACGCTCCTCATTGATGGT
Gria1	glutamate ionotropic receptor AMPA type subunit 1	AGAGGCTGGTGGTGGTTGACT	ACCCTGGTATGGTCTCGGGA
Gria2	glutamate ionotropic receptor AMPA type subunit 2	GGCGTGTAATCCTGGACTGT	ACACCAGGGAATCGTCGTAG
Gabrg2	gamma-aminobutyric acid type A receptor gamma 2 subunit	CGGAAACCAAGCAAGGATAA	ACAGTCCTTGCCATCCAAAC
Gabrd	gamma-aminobutyric acid type A receptor delta subunit	GCTGGACCTGGAGAGCTATG	CCGAAGCTGGAAGTGTAAGC
Gabra1	gamma-aminobutyric acid type A receptor alpha 1 subunit	TTGACTGTGAGAGCCGAATG	AAACGTGACCCATCTTCTGC
Gabra2	gamma-aminobutyric acid type A receptor alpha 2 subunit	CCATGCACTTGGAGGACTT	ACTGGCCCAGCAAATCATAC
Gapdh	glyceraldehyde-3-phosphate dehydrogenase	TCCCTGTTCTAGAGACAG	CCACTTTGTCACAAGAGA
Cnr1	cannabinoid receptor 1	GTCGATCCTAGATGGCCTTGC	GTCATTCGAGCCCACGTAGA G
Dagla	diacylglycerol lipase, alpha	CTTTGCTGAATTTTTCCGTGACC	TTGTTTGCCTCATCCAGCAC
Mgll	monoacylglycerol lipase	CTACCTGCTCATGGAATC	GACACCCACGTATTTATTTC
Napepl d	N-acyl phosphatidylethanolamin e phospholipase D	AGATATGGACTCAAGAGTGAAGAC TTC	TCCTCAAAGGCTTTGTCATCG G
Faah	fatty acid amide hydrolase	GTTACAGAGTGGAGAGCTGTCC	GTCTCACAGTCGGTCAGATA GG

2.1.6. Statistical analyses

We used two different approaches to study the relationship between gene expression and impulsivity: a clustering approach and a correlational approach.

For the clustering approach the animals were classified according to their impulsivity using hierarchical cluster analysis with Ward's method. Although other approaches, like a quartile categorisation, could be applied to isolate extreme sub-populations in our sample, we were interested in studying the whole population so that we could compare these results with those obtained in the correlational analysis (which must include the whole behavioural and neurochemical continuum of the entire population). We also refrained from using a quartile approach because doing so would incur in loss of power due to resulting smaller sample size.

We analysed the differences in the behaviour of the clustered groups with a two-way repeated-measures linear mixed models approach with either lever preference (for DDT) or premature responses (for 2-CSRTT) as the dependent variable, CLUSTER as the between-subject factor and DELAY or SESSION as the within-subject factor. We also used Student t-tests to test if the averages for *k* or the premature responses during the day of the test were significantly different between the clustered groups. Subsequently, we checked for statistical differences in gene expression between both groups using either the Student's t-test for the homocedastic and normal data or Mann Whitney's U when the parametric assumptions were not met. We applied a false discovery rate (FDR) correction using the Benjamini-Hochberg procedure with an FDR level of 0.1. We report Cohen's *d* as the effect size estimator for parametric and r for non-parametric data. All the uncorrected p values are available in the supplementary materials.

For the correlational approach, we measured the relationship between the expression of the genes with were found with differential expression between groups and either measure of impulsivity using Pearson's r when the populations of both variables were normally-distributed and Kendall's τ for the non-parametric data.

All the statistical analyses were performed using SPSS 24 (IBM) or InVivoStat (Bate and Clark, 2011) and the level of significance was set to α=0.05. All the graphs were designed using the PRISM 6 software (GraphPad Software, Inc) or Photoshop (Adobe Systems Inc.).

2.2. Results

2.2.1. Delay-discounting

Regarding impulsivity measured with the delay-discounting task, we used the k values of the animals to segregate them in two groups: 7 rats were assigned to the High Impulsive (HI-DD) group and 6 to the Low Impulsive (LI-DD) group (Figure 17A). As expected, HI-DD rats showed steeper discounting curves than LI-DD animals (significant CLUSTER*DELAY interaction ($F_{4, 44}$=7.48; p<0.001), significant effect of the CLUSTER factor ($F_{1, 11}$=12.57; p<0.01) and significant DELAY factor ($F_{4,44}$=51.56 p<0.0001) (Figure 17B). We also compared the average k value of both groups and verified that they differed significantly (t_{11}=-5.77; p<0.001; *d*=-3.16; Figure 17C).

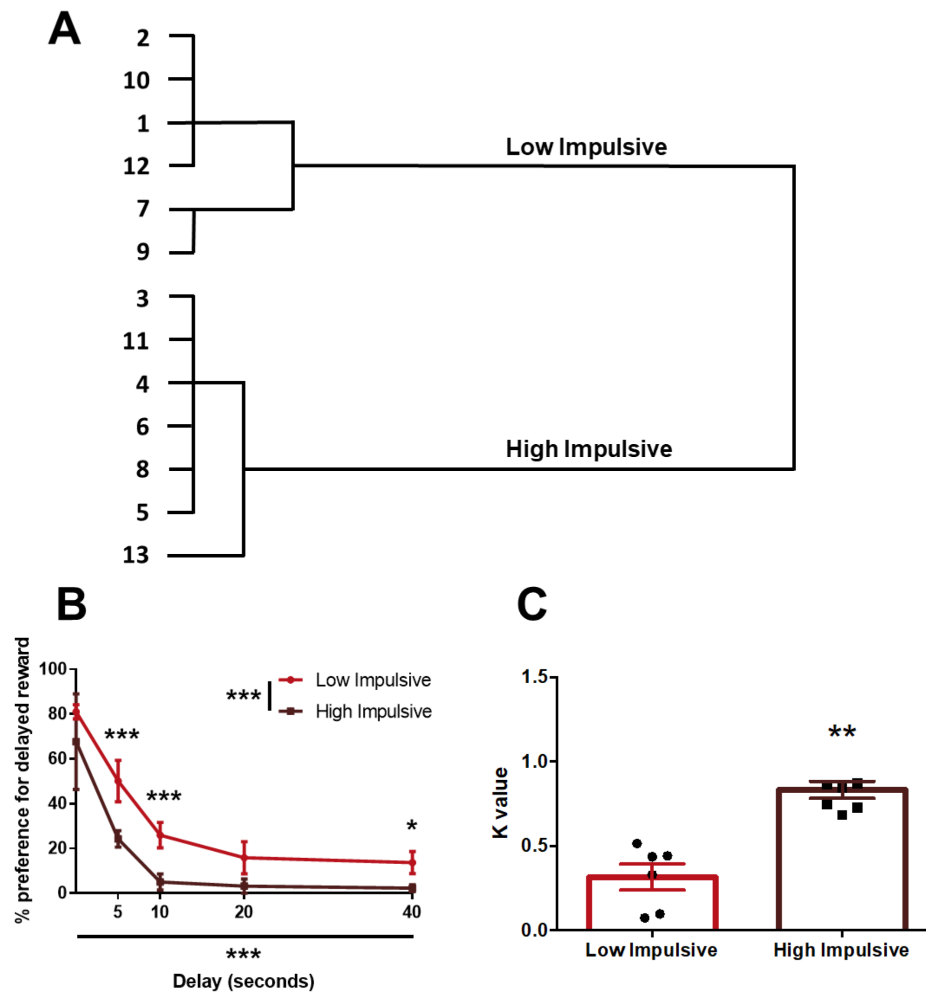


Figure 17: *Population segregation according to performance in the delay-discounting task.* ***A:*** *cluster analysis dendrogram showing the grouping of rats in high impulsive and low impulsive populations. Numbers correspond to the ID of each rat according to our numbering system for this experiment.* ***B:*** *delay discounting curves of high and low impulsive rats. * p<0.05 ** p<0.01 *** p<0.001 as compared to the low impulsive group. The main GROUP and DELAY effects are represented by the asterisks in the legend and the horizontal axis.* ***C:*** *k value of high impulsive and low impulsive animals. ** p<0.01 as compared to the low impulsive group. Line and bar graphs represent the mean ± standard error of the mean. Symbols in bar graphs represent individual data points from each rat.*

2.2.2. Two-choice serial reaction time

We also sorted another set of rats that performed the 2-CSRTT according to their premature responses in the long-ITI test day; they clustered in two groups: a high impulsive group of 11 rats (HI-2C) and a low impulsive group of 7 rats (LI-2C) (Figure 18A). The repeated measures linear mixed model analysis revealed no differences between both groups in either the premature, correct, incorrect, omitted or premature responses (Table 1 and Fig 18B). During the test, no differences were found between both groups in the number of omissions, incorrect or perseverative responses but the number of premature responses during the test was significantly different between both groups (t_{16}=-6,385; p<0.001; *d*=-3,07; Figure 18C).

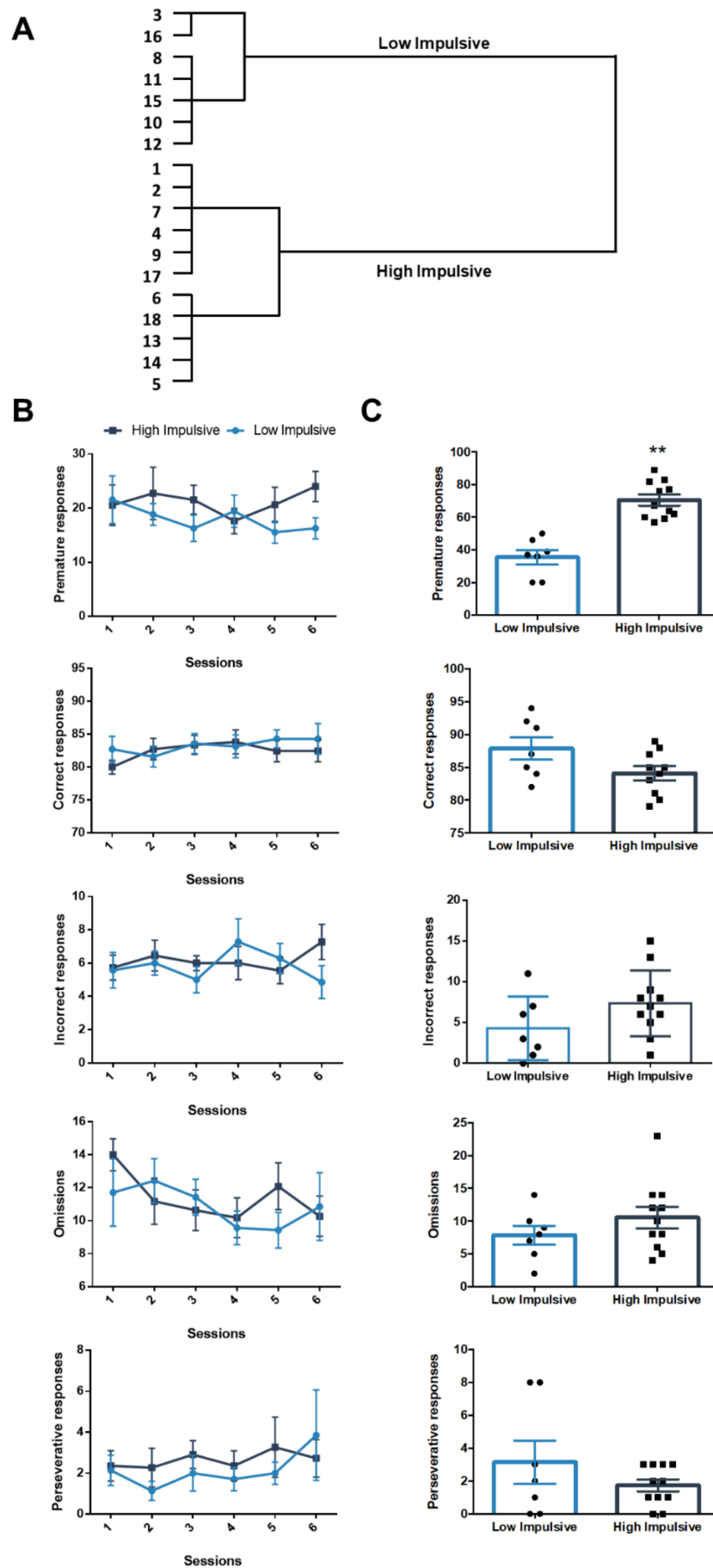


Figure 18: *Population segregation according to performance in the 2-CSRTT.* ***A:*** *cluster analysis dendrogram showing the grouping of rats in high impulsive and low impulsive populations. Numbers correspond to the ID of each rat according to our numbering system for this experiment. These numbers represent different rats from those used in the DDT experiment.* ***B:*** *performance in the 2-CSRTT during the last six sessions, prior to the test day.* ***C:*** *performance on the days of the test (ITI= 9 sec). ** p<0.01 as compared to the low impulsive group. Line and bar graphs represent the mean ± standard error of the mean. Symbols in bar graphs represent individual data points from each rat.*

2.2.3. Gene expression

Regarding impulsivity measured by the DDT, we found that the rats of the HI-DD group expressed higher levels of *Cnr1* in the mOFC than the rats of the LI-DD group (t_8=-4.13; p<0.01; d=-2.71; Figure 19A). We also found a significant positive correlation between k and the expression of *Cnr1* in the mOFC (r=0.77; p<0.01) and *Gria1* (r=0.65; p<0.05). Accordingly, the animals that expressed higher levels of expression of these genes displayed higher impulsivity in this task (Figure 19A).

The analysis of the differences between the groups extracted by cluster analysis by premature responding revealed that the expression of Gria1 in the lOFC was lower in the HI-2C as compared to LI-2C rats (t15=2.31; p<0.05; d=1.1; Figure 19B), and the same was true for Gabra1 in the lOFC (t15=3.19; p<0.01; d=1.79; Figure 19B). We also found that the premature responses during the test were inversely related to the expression of *Gabra1* in the lOFC (r=-0.48; p<0.05 uncorrected). The animals that expressed lower levels of *Gabra1* were less prone to make premature responses and hence, less impulsive (Figure 19B). There were no *Gabra1* gene expression differences between HI-2C and LI-2C in the mOFC.

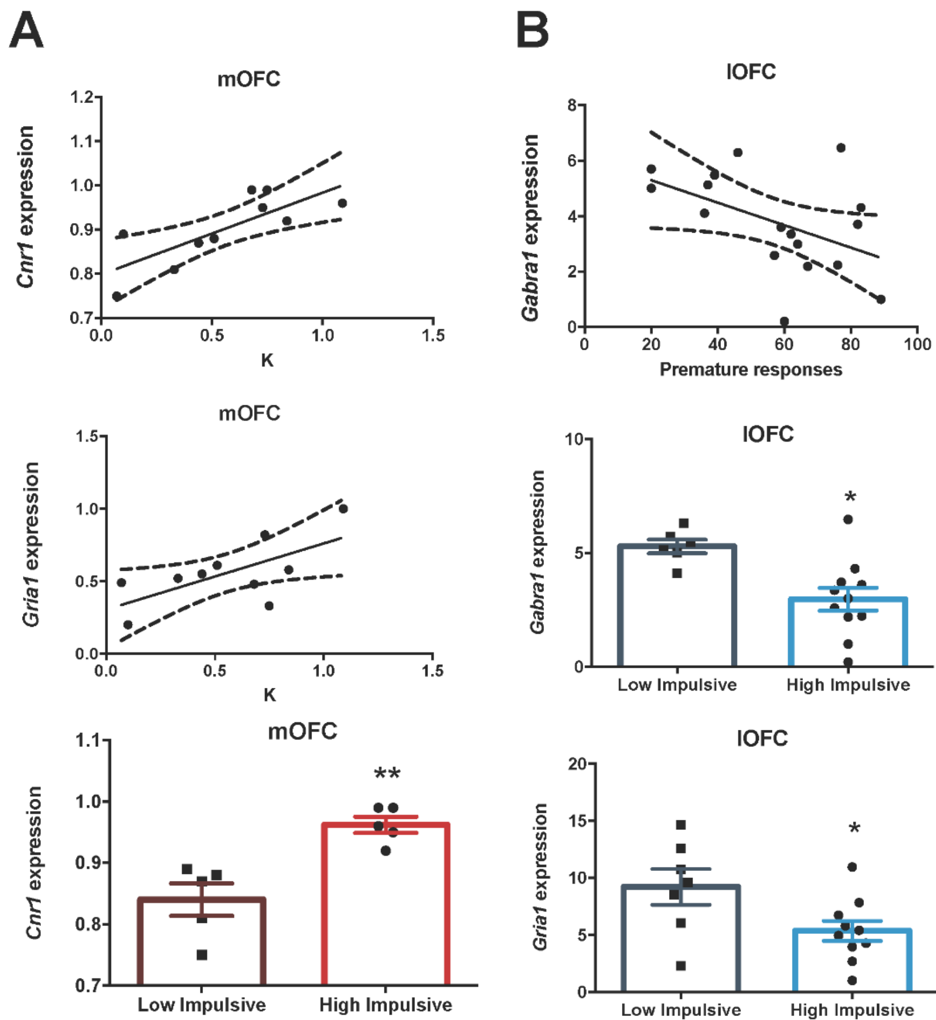


Figure 19: *The relationship between gene expression and impulsive behaviour. Gene expression of AMPA, NMDA and GABAA receptor subunits as well as of elements of the endocannabinoid system in the lateral and medial orbitofrontal cortex (lOF and mOFC, respectively) was assayed one week after impulsivity assessment.* ***A:*** *impulsive choice was positively correlated with Cnr1 (CB1 cannabinoid receptor) and Gria1 (GluA1 AMPA receptor subunit) gene expression the mOFC. Moreover, rats classified as high impulsive according to their delay discounting showed a significantly higher expression of the Cnr1 gene in the mOFC as compared to low impulsive rats.* ***B:*** *impulsive action in the 2-CSRTT was negatively correlated with the gene expression of the Gabra1 gene (which encodes the alpha 1 subunit of the GABAA receptor) in the lOFC. In addition, animals classified as high impulsive in the 2-CSRTT showed significantly lower levels of expression of the Gabra1 and Gria1 genes in the lOFC. Correlations are represented the best fit regression lines with dashed lines depicting the 95% confidence interval. Bar graphs represent the mean ± standard error of the mean. Symbols in bar graphs represent individual data points from each rat. * p<0.05 as compared to the low impulsive group.*

2.3. Discussion

This study was aimed at determining if the expression of certain genes related to glutamatergic, GABAergic or endocannabinoid neurotransmission was associated to two different components of waiting impulsivity (delay-discounting and premature

responding) and if there was neuroanatomical segregation between the medial and lateral divisions of the OFC in this relationship. For this purpose, we classified two separate groups of rats according to their performance in each task. A hierarchical clustering approach was chosen as the sorting strategy because, as observed from the figures, there was not a large variance between groups. We then compared the expression of selected genes related to neurotransmission in the medial and lateral orbitofrontal cortices between the resulting groups, searching for potential differences that could be specific to each variety of impulsivity.

Our results suggest that the gene expression signature of these two elements of waiting impulsivity is indeed different. We have found that, at the level of the genes studied here, the motor impulsivity component measured in the 2-CSRTT was mostly related to GABAergic gene expression in the lOFC, while the choice impulsivity assessed in the delay-discounting task was correlated with endocannabinoid gene expression in the mOFC.

The OFC has been strongly implicated in impulsiveness, goal-directed behaviour and decision-making processes, although its key role in these psychological phenomena has been recently challenged (Stalnaker et al., 2015). With regard to impulsive behaviour, the lesion studies that have been performed using delay-discounting task measurements of impulsive choice show conflicting results (Chudasama et al., 2003; Mar et al., 2011; Mobini et al., 2002; Rudebeck et al., 2006; Winstanley et al., 2004b). The functional heterogeneity in the OFC has been suggested to be one of the reasons for such discrepancies (Mar et al., 2011; Stopper et al., 2014).

The mOFC has been proposed to be a hub where the different value signals of subjective goals are integrated (Kable and Glimcher, 2009). Indeed, mOFC-lesioned monkeys have difficulty making choices when the value of two options is close (Noonan et al., 2010) and studies with human patients have shown that mOFC lesions affect reward valuation and self-control in intertemporal choice tasks (Peters and D'Esposito, 2016). Rat lesion studies also provide evidence for a role of the mOFC in impulsive choice whereby mOFC damage increases the preference for a large but delayed reward (Mar et al., 2011). We have found that expression of *Cnr1* in the mOFC was directly related to the waiting impulsivity that is captured by the delay-discounting task. The relationship between the endocannabinoid system and the different varieties of impulsivity is complex (see Moreira et al., (2015) for an excellent review). Some previous reports suggested that the activation of CB_1 receptors in the OFC promote impulsive choice (Fatahi et al., 2018; Khani et al., 2015). However, these studies mainly targeted the lateral and ventral divisions of the OFC, making any comparison to the present results problematic. There are also previous studies assessing the effects of

systemic injections of CB_1 receptor agonists that suggest that THC administration reduced choice impulsivity measured with the delay-discounting task (Wiskerke et al., 2011). Interestingly, another study showed no effect after treatment with a cannabinoid agonist WIN 55,512-2 (Pattij et al., 2007). It is important to note that CB_1 receptors are mostly presynaptically localized in axon terminals, so the gene expression differences found here (arising from mRNAs in the cell bodies) could be modulating neurotransmission distally, in terminal areas such as the hippocampus, a structure that is strongly connected to the mOFC (Fettes et al., 2017). In any case, the higher levels of *Cnr1* gene expression in high impulsive animals in the mOFC may suggest that this subpopulation could be especially vulnerable to the disrupting effects of cannabinoids on those cognitive processes that depend on the normal function of the mOFC, such as reward valuation or self-control. It could also mean that, based on their differential expression of cannabinoid receptors, high impulsive individuals might reduce their impulsivity (or at least the tolerance to delay component of impulsivity) to a higher degree than low impulsive individuals, after marihuana use. This hypothesis merits further testing.

Previous studies, both in humans (Elliott et al., 2000) and monkeys (Iversen and Mishkin, 1970; Noonan et al., 2010) have shown that the lOFC is specifically required when a response previously associated with reward has to be suppressed (but see Gourley et al., 2010) and, conversely, its inactivation leads to impaired adjustment of behaviour after non-rewarded actions (Dalton et al., 2016). While lesions of the lOFC have been shown to increase impulsive choice (Mar et al., 2011), to the best of our knowledge, a clear (and specific) role for the lOFC in premature responding has not yet been established.

Gabra1 expression was lower in the animals that made more premature responses in the 2-CSRTT. In forebrain pyramidal neurons, $GABA_A$ receptors containing the alpha 1 subunit are mainly expressed throughout the somatodendritic region while those containing the alpha 2 subunit are mostly localised to the initial axon segment (Loup et al., 1998; Nusser et al., 1996). This differential expression of the subunit in high and low impulsive animals could translate into net differences in the cellular localisation of the receptor in both populations and this might have implications for how inhibitory signals are integrated by the cortical pyramidal neurons where these receptors are expressed. There are other previous studies that have involved the GABAergic system in impulsive action. For example, Jupp and colleagues found that $GABA_A$ binding in the anterior cingulate cortex was negatively correlated with premature responding in the 5-CSRTT (Jupp et al., 2013) and Caprioli and co-workers established a role of the GABA synthesis enzyme GAD (glutamic acid decarboxylase) within the nucleus accumbens core in premature responding (Caprioli et al., 2014) In addition, GAD inhibition in the

medial prefrontal cortex impaired impulse control measured in the 5-CSRTT (Paine et al., 2015).

Contrary to the direct relationship between delay discounting and *Gria1* expression in the mOFC, high impulsive animals in the 2CSRTT show lower expression levels of this gene in the lOFC. The fact that *Gria1* but not *Gria2* is potentially involved in both measures of impulsivity (although in different areas and in opposed directions) is of note. Most of the AMPA receptors in the brain are heterotetramers with different combinations of GluA1 and GluA2. The edited transcript of GluA2 (which represents >99% of the transcripts in the brain) renders the receptor impermeable to Ca^{2+}, but AMPA receptors lacking this subunit allow this cation flow when open (Egebjerg et al., 1994; Sommer et al., 1991). This GluA2-lacking receptors, which are tipically assembled from four GluA1 subunits, apparently have a preeminent role in mediating changes in synaptic plasticity (Cull-Candy et al., 2006; Wiltgen et al., 2010). Whether the expression levels of *Gria1* in the mOFC and lOFC is related with the role of those areas in impulsivity would need further studies. In our study, the high impulsive group of rats that performed the 2CSRTT have lower expression of *Gria1* in the lOFC, and this is consistent with previous findings. Indeed, GluA1 blockade has been linked to high impulsivity scores in the 2CSRTT that reverted to normal after the administration of an AMPA receptor agonist, suggesting that AMPA receptors are tonically implicated in the regulation of impulsive behaviour (Nakamura et al., 2000). With regard to impulsive choice, GluA1 knock-out mice are impulsive in a T-maze task designed to measure impulsive choice (Barkus et al., 2012) and also in a differential reinforcement of low rates task (Reisel et al., 2005).

In conclusion, we here provide the first evidence for a dissociation between the medial and lateral division of the OFC in impulsive action and impulsive choice and suggest that CB_1 and AMPA receptors in the mOFC are positively coupled to the expression of impulsive choice while $GABA_A$ and AMPA receptors in the lOFC are markers of impulsive action. Functional studies interfering with or augmenting the expression of these genes must now be conducted in order to ascertain if there is a causal relationship between the gene transcription variations here reported and the different varieties of waiting impulsivity that we have studied in this work.

Chapter 3

Study on the effects morphine self-administration and subsequent extinction in the expression of genes related to the mTOR network

Ucha et al., 2019a

3.1. Materials and methodsAnimals

Adult male Lewis rats (Charles River Laboratories) were housed in groups of 4 in plastic cages with wood chips bedding inside of a temperature and humidity-controlled facility, and on a 12h/12h light/dark cycle (lights on at 8:00am) with ad libitum access to food (standard commercial rodent diet A04/A03: Panlab) and water. Animals were allowed at least one week to acclimatise to the animal facility and they weighed around 250-300 g when the experimental procedures commenced. All the animals were maintained and handled according to European Union guidelines for the care of laboratory animals (EU Directive 2010/63/EU governing animal experimentation) and the Ethical Committee of UNED approved all the experimental procedures.

3.1.2. Experimental groups

Animals were randomly assigned to the following groups: Morphine Self-administration (MSA), Vehicle Self-administration (VhSA), Morphine Extinction (MEx) and Vehicle Extinction (VhEx). Due to the limited number of operant boxes, several iterations of the self-administration experiments with animals from each of the four groups were performed until a minimum of 8 subjects per group was obtained. Four animals were excluded from the experiment due to the loss of the skull mount or catheter patency issues.

3.1.3. Apparatus

Twelve operant conditioning chambers (l=300mm; w=245mm; h=328mm) (Coulborne Instruments), each equipped with a pellet dispenser and a microliter injection pump, were used for the morphine self-administration and extinction studies. A catheter was connected to the rat and held in place with a spring-tether system, and a rotating swivel, which allowed the animals to move freely inside the chamber. Two levers placed 14cm apart were available throughout all the sessions, one of them inactive. Due to a technical issue with the MedState program, the responses of the inactive lever were not recorded.

3.1.4. Experimental protocol

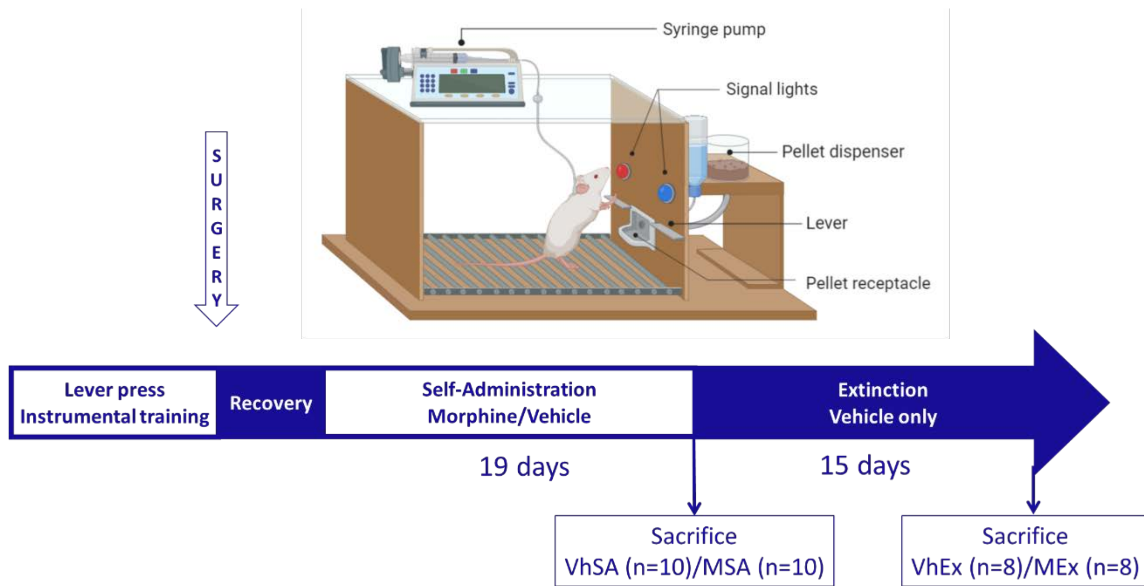


Figure 20: *Experimental schedule for this experiment and setup of the conditioning chamber used for all the phases.*

3.1.4.1. Lever press instrumental training

At the beginning of the experiment, all the rats received daily instrumental training sessions with food pellets as reinforcers (grain-based rodent tablet, Testdiet™) on a fixed ratio 1 schedule, facilitating the acquisition of self-administration behaviour. During this training, the rats had restricted access to food (14 grams/day). The sessions lasted 30 minutes and continued until the animals developed a robust lever press behaviour (at least 100 lever presses in three consecutive training sessions).

3.1.4.2. Surgery

Rats were anaesthetized with an isoflurane/oxygen mixture (5% isoflurane during induction; 2% ±0.5% for maintenance), and a polyvinyl chloride catheter (0,16mm i.d.) was inserted into the right jugular vein of the animal approximately at the level of the atrium and secured there with silk thread knots. The catheter was fixed subcutaneously around the neck, exiting the skin at the midscapular region. A pedestal of dental cement was then mounted on the skull of the rat in order to attach the tethering system. After surgery, the rats were allowed to recover for 7 days and a nonsteroidal anti-inflammatory drug (NSAID) (meloxicam - Metacam™: 15 drops of a 1.5 g/ml solution per 500 ml of water) was added to the drinking water. Until the end of the self-administration procedure, the catheters were flushed daily with a sterile saline solution containing sodium heparin (100 IU/ml) and gentamicin (1mg/ml) to maintain catheter patency and to prevent infections.

3.1.4.3. Morphine self-administration

A week after recovering from surgery, the rats underwent 19 daily sessions of morphine self-administration. During the dark phase of the light cycle, for 12 hours (starting at 8 pm) rats were allowed daily access to morphine (1 mg/kg in a sterile saline -0.9% NaCl- solution) or its vehicle alone under a fixed-ratio 1 reinforced schedule. During these sessions, one active lever press resulted in morphine infusion (1 mg/kg morphine in saline solution delivered over 10 seconds) followed by a 10-second time-out. A light cue located above the active lever indicated the availability of the drug, only being turned off during drug delivery, time out and at the end of each session. A limit of 50 infusions per session was set in order to avoid overdosing. One day after the last session, two groups of rats were sacrificed (VhSA, n=10; MSA, n=10), and their brains were processed and stored.

Another challenge in addiction research is to understand how the consumers shift from controlled recreational use of drugs to pathological compulsive behaviour. This shift is usually accompanied by a gradual escalation in drug intake (Edwards, 1986; Gawin, 1991; Marlatt et al., 1988), which has been replicated in animal models of self-administration. Rats trained to self-administer drugs under extended access conditions (usually >6 hours/day depending on the drug; the typical restricted access conditions are 1-2 hours/day) show this escalation phenomenon (Ahmed and Koob, 1998; Kitamura et al., 2006; Picetti et al., 2010). Moreover, rats under extended access show increased seeking motivation in progressive ratio tests (Lenoir and Ahmed, 2008; Wee et al., 2007) and continue self-administering despite being punished (Vanderschuren and Everitt, 2004).

In our opioid studies, we have decided to use extended access conditions (Morphine: 12h/sessions; Heroin: 6h/sessions) because they have been suggested to better reproduce some of the clinical aspects of addiction in humans

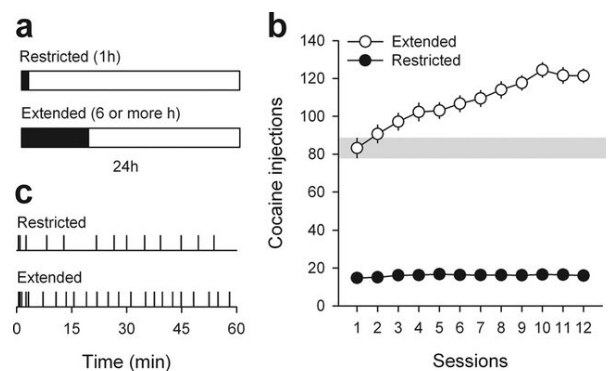


Figure 21: *Differences between restricted and extended access in cocaine self-administration shown in the studies of Serge H. Ahmed. a) After acquisition of cocaine self-administration, one group of rats has restricted access to cocaine during only 1 h per day, while the other experimental group has extended access to cocaine during six. (b) Escalation of cocaine intake in rats with extended drug access. The horizontal grey box indicates the mean number (SEM) of drug injections during the first day. (c) First-hour distribution of cocaine injections by two representative individual rats., showing that escalation of cocaine intake is largely due to acceleration in the rate of cocaine self-administration. (Ahmed, 2011)*

Box 3: *Extended access*

3.1.4.4. Extinction training

The remaining rats were given 15 daily sessions of extinction training. The protocol was the same as in the self-administration sessions, but in this phase, all the rats received saline injections instead of morphine. One day after the last extinction session, the two remaining groups of rats (VhEx, n=8; MEx, n=8) were sacrificed, and their brains processed and stored.

3.1.5. Sample processing

On the day of the sacrifice, the rats were decapitated and with the help of a brain matrix, 1 mm thick coronal slices were obtained at approximately 4.2mm anterior from bregma for the prefrontal cortex, at approximately 3.10 mm posterior from bregma for the amygdala and at approximately 1.70 mm posterior from bregma for the PFC. With the help of two dissecting lancet-shaped needles, the amygdala (mainly the basolateral amygdala – BLA, although some marginal amounts of the adjacent central amygdala might have been included in some cases), the NAcc (both shell and core) and the prefrontal cortex (mostly the orbitofrontal cortex, OFC, although some marginal amounts of the agranular insular cortex might have been included in some cases) were dissected according to the Paxinos and Watson atlas (Franklin and Paxinos, 2007) (see Figure 22).

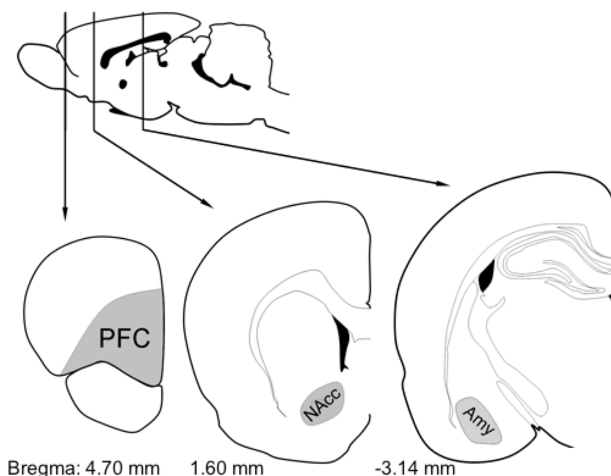


Figure 22: *Schematic representation of the sections of the rat brain with the areas dissected out highlighted in grey.*

All the surfaces and tools used for dissection were sterilised and treated with RNAseZap® (Ambion™), and all the steps were carried out with caution to maintain RNA integrity. The tissue samples from one hemisphere (randomised) were preserved overnight at 4 ºC in RNAlater® (Ambion™) and then stored at -70 ºC in RNAlater® for later RT-qPCR analysis. The samples of the other hemisphere were snap-frozen with dry ice and stored at -70º for western blot analysis.

3.1.6. RT-qPCR analysis

The samples stored in RNAlater® were homogenised in QIAzol lysis reagent (QIAgen) using a pellet pestle. The total RNA was extracted and precipitated using the chloroform, isopropanol and ethanol method (Chomczynski and Sacchi, 1987) with

glycogen as a carrier. The precipitate was dissolved in RNAse free water, and the concentration and RNA integrity (as indexed by the RNA integrity number [RIN] value) was assessed in a bioanalyzer (Agilent 2100). The RNA concentration in each sample was adjusted by adding RNAse free water and to avoid genomic DNA contamination, DNAse digestion was performed (DNAse I, Amplification Grade, Invitrogen) following the manufacturer's instructions. Finally, the samples were retrotranscribed using a commercial kit (Biorad iScript™ cDNA Synthesis Kit). PCR assays were performed on a real-time PCR detection system (CFX9600, Biorad) with an SSO Advanced SYBR mix (Biorad) using the primers indicated in the table 6. We ran duplicates of all the samples along with a no-template control and a no-RT control. We discarded the data of any assay with an unusual amplification or melt curve, if the difference between them was between duplicates was higher than one cycle. The relative expression of each gene calculated as described in Pfaffl, 2001 using Gapdh as a reference gene and the reaction efficiencies were obtained using LinRegPCR software (Ruijter et al., 2009), and normalised to the VhSA group.

***Table 6:** List of primers pairs used for the RT-qPCR asssays*

Gene	Primer sequences (5'-3')	
	Sense	Antisense
Akt1	CGCTTCTTTGCCAACATCGT	TCATCTTGATCAGGCGGTGT
Akt2	GGCACGCTTTTATGGAGCAG	ATCTCGTACATGACCACGCC
Eef1a1	TTGGACACGTAGATTCCGGC	TAGTGATACCACGCTCACGC
Eif4e	TACAGAACAGGTGGGCACTC	CATCGTCCTCCCCGTTTGTT
Gapdh	TCCCTGTTCTAGAGACAG	CCACTTTGTCACAAGAGA
Gsk3a	GCCCAACGTGTCCTACATCT	TTGGCGTCCCTAGTACCTTG
Gsk3b	CCGAGGAGAGCCCAATGTTT	CTTCGTCCAAGGATGTGCCT
Igf1r	ATCTCCGGTCTCTAAGGCCA	CCAGGTCTCTGTGGACGAAC
Igf2r	TCACAATCGAGGTGGACTGC	CACCCGGTGACAGACATTGA
Insr	GCTTCTGCCAAGACCTTCAC	TAGGACAGGGTCCCAGACAC
Mtor	GGTGGACGAGCTCTTTGTCA	AGGAGCCCTAACACTCGGAT
Rp6kb1	ACTGGAGCACCTCCATTCAC	GCTTGGACTTCTCCAGCATC
Pdk1	GAAGCAGTTCCTGGACTTCG	GCTTTGGATATACCAACTTTGTACC
Pik3ca	GAGCACAGCCAAGGAAACTC	TCTCCCCAGTACCATTCAGC
Rptor	CTTGGACTTGCTGGGACGAT	ATGAAGACAAGGAGTGGCCG
Rictor	CCGTCGCAGCAATCAAAGAC	CCCCCAATTCGATGAGCCAA
Rps6	CGTCTTGTTACTCCCCGTGT	GCCTACGTCTCTTGGCAATC
Sgk1	TGGTAGCAATCCTCATCGCTTT	GTGAGGGGTTGGCGTTCATA
Eif4ebp2	TCCTGGCGCCTTAATGGAAG	AAGATGTGGCTGGACAGAGC

3.1.7. Western blotting

The tissue samples were homogenised using a pellet pestle in 10 volumes of lysis buffer: 50mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid (HEPES) [pH7.5], 320 mM sucrose, (CompleteTM ethylenediaminetetraacetic acid [EDTA]-free, Roche) protease inhibitors, and phosphatase inhibitors (PHOStopTM, Roche). The resulting homogenate was centrifuged at 2000 g and at 4 ºC for 10 minutes, the supernatants were recovered and their protein concentration was assessed using the Bradford assay (Bio-Rad Protein Assay). The protein extracts (3 g) were mixed with 6X Laemmli buffer and loaded onto 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels, resolved by electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes. After blocking non-specific interactions with 5% bovine serum albumin (BSA) for one hour, the membranes were probed overnight with the primary antibodies (see Table 7) that were then recognized with a horseradish peroxidase-conjugated secondary antibody (see supplementary materials). Antibody binding was visualized by electrochemiluminescence (ECL Plus Western Blotting Substrate, Pierce™). As a control for protein loading, we measured the total protein loaded by adding 2,2,2-trichloroethanol to the gels prior to polymerization (final concentration 0.5% v/v: Ladner et al., 2004), and after resolving the gel, it was excited with an ultraviolet (UV) transilluminator and the fluorescence emitted was measured. We used a charge-coupled device (CCD) based detector (Amersham Imager 600) to capture both the chemiluminescence and the UV/fluorescence images, and the ImageJ software to analyze and quantify them. When necessary, antibodies were stripped using a harsh stripping protocol ("Stripping for reprobing": Abcam®).

Table 7: *List of antibodies used in western blot assays*

Target	**Phosphorylation site**	**Species**	**Company**	**Reference number**
Rabbit IgG	N/A	Goat	Abcam	ab6721
Phospho-PDK1	Ser241	Rabbit	Cell Signalling	3061
Phospho-mTOR	Ser2448	Rabbit	Cell Signalling	5536
Phospho-p70 S6 Kinase	Thr389	Rabbit	Cell Signalling	9205
Phospho-Akt	Ser473	Rabbit	Cell Signalling	4060
Phospho-GSK-3α/β	Ser21/9	Rabbit	Cell Signalling	9331

3.1.8. Statistical analysis

The data obtained from the self-administration and extinction experiments were analysed using repeated-measures ANOVA. The analysis of the self-administration data had Sessions as a within-subject factor and Treatment (Morphine-M- or Vehicle-Vh-) and Phase (Self-administration-SA- or Extinction-Ex-) as between-subject factors. The factor Phase was included in order to verify that there were no differences in self-administration behaviour (i.e. that the self-administration curves were comparable) between the rats used to analyse self-administration effects and those used to analyse extinction-related alterations. In the analysis of the extinction behavioural data, we only examined the effects of Treatment (between-subject factor) and Sessions (within-subjects factor). The degrees of freedom were adjusted by applying the Greenhouse-Geisser correction when the sphericity assumption was violated.

To analyze the biochemical assays two-way ANOVAs were performed with two between-subject factors: Treatment and Phase. When the required assumptions for ANOVA were not met, logarithmic, square root or reciprocal transformations were applied. If the assumptions were still violated, a Kruskal-Wallis test was performed followed by a multiple comparison of mean rank sums with VhSA as the control condition including a Bonferroni correction to the p-values (Conover, 1999).

Effect sizes were calculated for all the significant results, eta squared for the ANOVAs (η^2), generalised eta squared for the repeated measures ANOVAs (ηG^2) (Bakeman, 2005) and chi-squared for Kruskal-Wallis analyses.

3.1.9. Software

The statistical analyses were performed using SPSS 24 (IBM), and the level of significance was set to α=0.05 (uncorrected). The non-parametric multiple comparisons of groups were implemented in R, using the kwManyOneConoverTest function of the PMCMRPlus package (https://CRAN.R-project.org/package=PMCMRplus) by Thorsten Pohlert. All the graphs were designed using the PRISM 6 software (GraphPad Software, Inc).

3.2. Results

3.2.1. Behavioural data

All the animals achieved a high number of active lever presses during the acquisition phase, probably due to the previous autoshaping training (Fig. 1). Subsequently, the rats that received saline lowered the rate of active lever pressing, whereas the number of active lever presses of the rats that received morphine remained high. During the first extinction session, there was a surge in the number of

active lever presses in the rats of the MEx group, although this decreased gradually in the following sessions until it reached values similar to those of the VhEx group. The two way-repeated measures ANOVA showed a significant effect of the Sessions factor ($F_{7.34,227.63}$=3.94, p<0.001, ηG^2=0.07). We also found a significant effect of the Treatment factor ($F_{1,31}$=73,42, p<0.001, η2=0.7) suggesting that MSA animals pressed more the active lever than VhSA rats throughout the self-administration sessions. We did not find any significant Treatment*Phase interaction ($F_{1,31}$=0,425, p=0.52, η2=0.004) or any effect of the Phase factor ($F_{1,31}$=0,276, p=0.6, η2=0.002). Therefore, it was concluded that the groups that underwent extinction performed similarly to their counterparts during the self-administration procedure. Regarding the extinction session data, we found a significant effect of the Sessions factor ($F_{5.71,74.28}$=3,67, p=0.003, ηG^2=0.17). We also found a significant effect of the Treatment factor ($F_{1,13}$=12.02, p=0.004, η2=0.48) for the average values throughout the extinction sessions. To test whether the rats in the MEx group had extinguished the morphine self-administration behaviour, we compared the mean number of active lever presses during the last three days of extinction in the MEx and VhEx groups. Importantly, no significant differences were observed between these groups of rats (t_{14}=-1.71, p>0.05).

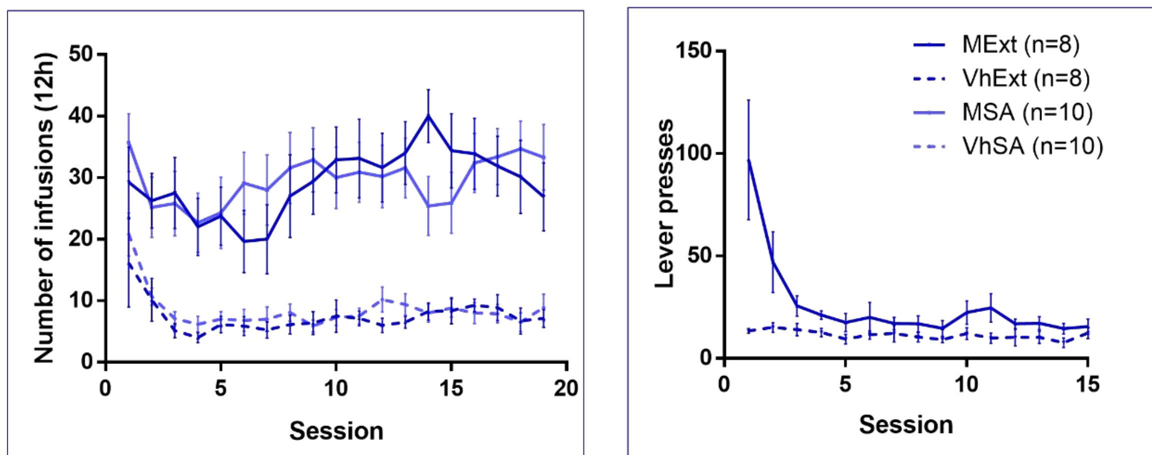


Figure 23: *Graphical representation of the behavioural data (VhSA - Vehicle self-administration; MSA - Morphine self-administration; VhEx - Vehicle extinction; MEx -Morphine extinction).*

3.2.2. Gene expression

Most of the RIN values obtained ranged from 7 to 9. In some very rare exceptions we obtained lower values, but in those cases, we verified that the Cts of the GAPDH expression were in the same range as those of the other samples in the group. In the amygdala, the gene expression analysis identified a significant effect of the treatment on the expression of the Regulatory Associated Protein of MTOR Complex 1 (*Rptor*) ($F_{1,28}$=5.57, p=0.025, η2=0.16) and the Eukaryotic Translation Initiation Factor

PFC				
Gene	**VhSA**	**MSA**	**VhEx**	**MEx**
Akt1	1 ± 0.195	0.994 ± 0.242	0.998 ± 0.079	0.994 ± 0.107
Akt2	1 ± 0.566	0.946 ± 0.408	0.992 ± 0.79	1.146 ± 0.486
Eef1a1	1 ± 0.504	0.81 ± 0.35	0.689 ± 0.33	0.844 ± 0.378
Eif4e	1 ± 0.621	0.725 ± 0.189	0.643 ± 0.327	1.11 ± 0.634
Gsk3a	1 ± 0.415	0.867 ± 0.262	0.882 ± 0.554	0.971 ± 0.33
Gsk3b	1 ± 0.706	1.023 ± 0.479	0.75 ± 0.454	1.025 ± 0.38
Igf1r	1 ± 0.159	1.103 ± 0.191	0.987 ± 0.147	1.171 ± 0.299
Igf2r#	1 ± 0.941	1.467 ± 1.091	0.363 ± 0.166	0.504 ± 0.387
Insr	1 ± 0.365	0.85 ± 0.317	0.938 ± 0.558	1.015 ± 0.372
Mtor	1 ± 0.481	1.127 ± 0.328	1.236 ± 0.818	1.339 ± 0.539
Rp6kb1	1 ± 0.397	0.892 ± 0.4	0.892 ± 0.461	0.948 ± 0.279
Pdk1	1 ± 0.271	0.93 ± 0.168	0.954 ± 0.463	1.194 ± 0.24
Pik3ca	1 ± 0.497	0.881 ± 0.409	0.872 ± 0.343	1.051 ± 0.286
Rptor	1 ± 0.343	0.961 ± 0.208	1.061 ± 0.298	1.138 ± 0.36
Rictor	1 ± 0.522	0.881 ± 0.269	0.687 ± 0.374	0.874 ± 0.393
Rps6	1 ± 0.296	0.969 ± 0.275	1.049 ± 0.371	1.065 ± 0.38
Sgk1	1 ± 0.395	1.617 ± 0.959	0.966 ± 0.412	1.091 ± 0.252
Eif4ebp2	1 ± 0.315	1.09 ± 0.423	1.117 ± 0.373	1.278 ± 0.191
NAcc				
Gene	**VhSA**	**MSA**	**VhEx**	**MEx**
Akt1	1 ± 0.31	0.851 ± 0.463	1.082 ± 632	1.184 ± 0.181
Akt2	1 ± 0.488	0.622 ± 0.518	0.983 ± 0.91	1.371 ± 0.625
Eef1a1	1 ± 0.427	0.86 ± 0.579	0.894 ± 0.687	1.04 ± 0.614
Eif4e	1 ± 1.303	2.605 ± 2.928	0.934 ± 1.227	0.851 ± 0.709
Gsk3a	1 ± 0.519	0.65 ± 0.408	0.662 ± 0.439	0.723 ± 0.401
Gsk3b	1 ± 0.432	0.849 ± 0.781	0.809 ± 0.569	0.905 ± 0.492
Igf1r	1 ± 0.358	0.915 ± 0.459	1.165 ± 0.767	1.096 ± 0.465
Igf2r	1 ± 0.524	0.585 ± 0.394	0.882 ± 0.587	0.971 ± 0.457
Insr	1 ± 0.372	0.848 ± 0.474	0.964 ± 0.789	0.963 ± 0.524
Mtor	1 ± 0.498	0.638 ± 0.501	0.777 ± 0.672	0.973 ± 0.499
Rp6kb1	1 ± 0.472	1.003 ± 0.386	1.192 ± 0.349	1.109 ± 0.343
Pdk1	1 ± 1.132	2.728 ± 1.947	1.038 ± 1.105	2.224 ± 2.518
Pik3ca	1 ± 0.655	0.921 ± 0.805	0.926 ± 0.697	1.1 ± 0.484
Rptor	1 ± 0.469	0.862 ± 0.774	1.016 ± 0.84	1.093 ± 0.816
Rictor	1 ± 0.476	0.751 ± 0.633	0.784 ± 0.55	0.935 ± 0.512
Rps6	1 ± 0.556	0.835 ± 0.688	1.301 ± 0.894	1.156 ± 0.748
Sgk1	1 ± 0.847	3.083 ± 3.071	1.195 ± 1.067	1.421 ± 1.33
Eif4ebp2	1 ± 0.484	0.756 ± 0.568	1.013 ± 0.637	1.248 ± 0.729
Amy				
Gene	**VhSA**	**MSA**	**VhEx**	**MEx**
Akt1#	1 ± 0.501	1.051 ± 0.537	1.552 ± 0.387	1.383 ± 0.442
Akt2	1 ± 0.512	1.369 ± 0.579	1.323 ± 0.554	1.324 ± 0.419
Eef1a1	1 ± 0.542	1.159 ± 0.403	1.244 ± 0.382	1.216 ± 0.348
Eif4e	1 ± 0.643	1.086 ± 0.488	1.042 ± 0.36	1.276 ± 0.611
Gsk3a	1 ± 0.576	1.118 ± 0.362	1.302 ± 0.447	1.166 ± 0.306
Gsk3b	1 ± 0.732	1.104 ± 0.764	1.41 ± 0.547	1.544 ± 1
Igf1r	1 ± 0.45	1.37 ± 0.478	1.42 ± 0.338	1.281 ± 0.468
Igf2r#	1 ± 0.362	1.378 ± 0.377	1.526 ± 0.31	1.498 ± 0.451
Insr[a]	1 ± 1.077	0.13 ± 0.068	0.095 ± 0.85	0.27 ± 0.152
Mtor	1 ± 0.625	1.023 ± 0.399	1.33 ± 0.406	1.432 ± 0.647
Rp6kb1	1 ± 0.364	1.124 ± 0.252	1.22 ± 0.187	1.197 ± 0.318
Pdk1	1 ± 0.608	1.421 ± 0.365	1.475 ± 0.138	1.29 ± 0.238
Pik3ca	1 ± 0.771	0.821 ± 0.282	0.947 ± 0.245	0.889 ± 0.259
Rptor*	1 ± 0.669	1.72 ± 0.77	1.368 ± 0.356	1.715 ± 0.64
Rictor	1 ± 0.503	1.301 ± 0.452	0.975 ± 0.114	1.203 ± 0.419
Rps6	1 ± 0.497	0.824 ± 0.244	0.631 ± 0.033	0.802 ± 0.276
Sgk1	1 ± 0.53	2.285 ± 1.382	1.282 ± 0.512	1.371 ± 0.516
Eif4ebp2*	1 ± 0.394	1.402 ± 0.518	1.23 ± 0.266	1.465 ± 0.497

Table 8: *Mean and standard deviation of the relative expression of the genes studied normalised to VhSA values. #Significant effect of the Phase factor ($p < 0.05$). *Significant effect of the Treatment factor ($p < 0.05$).*

4E Binding Protein 2 (Eif4ebp2) ($F_{1,28}$=4.28, p=0.048, η2=0.13: Table 1). The expression of these genes increased in the rats that self-administered morphine and this effect persisted even after extinction training. In this structure, we also found a main effect of the Phase factor on the expression of AKT Serine/Threonine Kinase 1 (*Akt1*) ($F_{1,28}$=6.9, p=0.014, η2=0.19) and the Insulin-Like Growth Factor 2 Receptor (*Igf2r*) ($F_{1,28}$=5.74, p=0.024, η2=0.15). In both cases transcription was enhanced after the extinction sessions. Significant differences in the Insulin Receptor (*Insr*) expression were evident between the four groups (χ^2_3=14.96, p<0.002) and the multiple comparison tests showed that the VhSA rats expressed *Insr* more strongly than the MSA and VhEx rats.

Igf2r expression was also affected In the PFC by the Phase factor ($F_{1,26}$=7.32, p=0.012, η2=0.21), although its expression was weaker after the extinction sessions.

There were no statistically significant differences in the expression of any of the genes analysed in the NAcc.

3.2.3. Phosphoprotein levels

We did not find any significant effects of the Treatment on the phosphoproteins assessed in each of the brain areas examined. However, in the amygdala the Phase factor affected the levels of phospho-GSK-3α (Ser21/9) ($F_{1,28}$=5.32, p=0.029, η2=0.14) and the 68kDa band of phospho-PDK1 (Ser241) ($F_{1,29}$=6.18, p=0.019, η2=0.17). The levels of both these phosphoproteins were lower after the extinction sessions (Figure. 26).

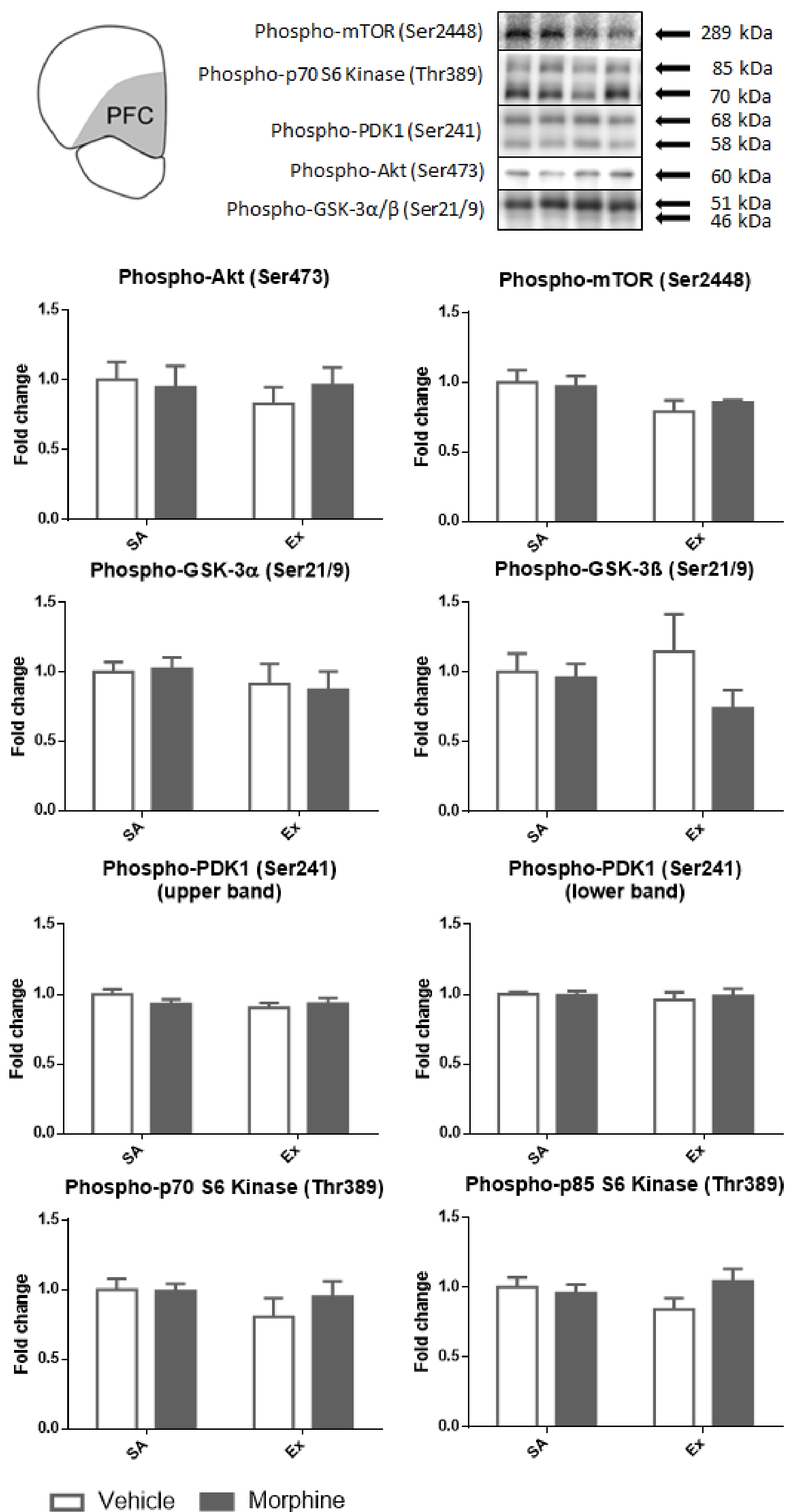


Figure 24: *Representative Western Blots to analyse phosphoproteins in the PFC, normalising the data to the total protein in the gel and the mean of the VhSA group (expressed as the mean ± SD).*

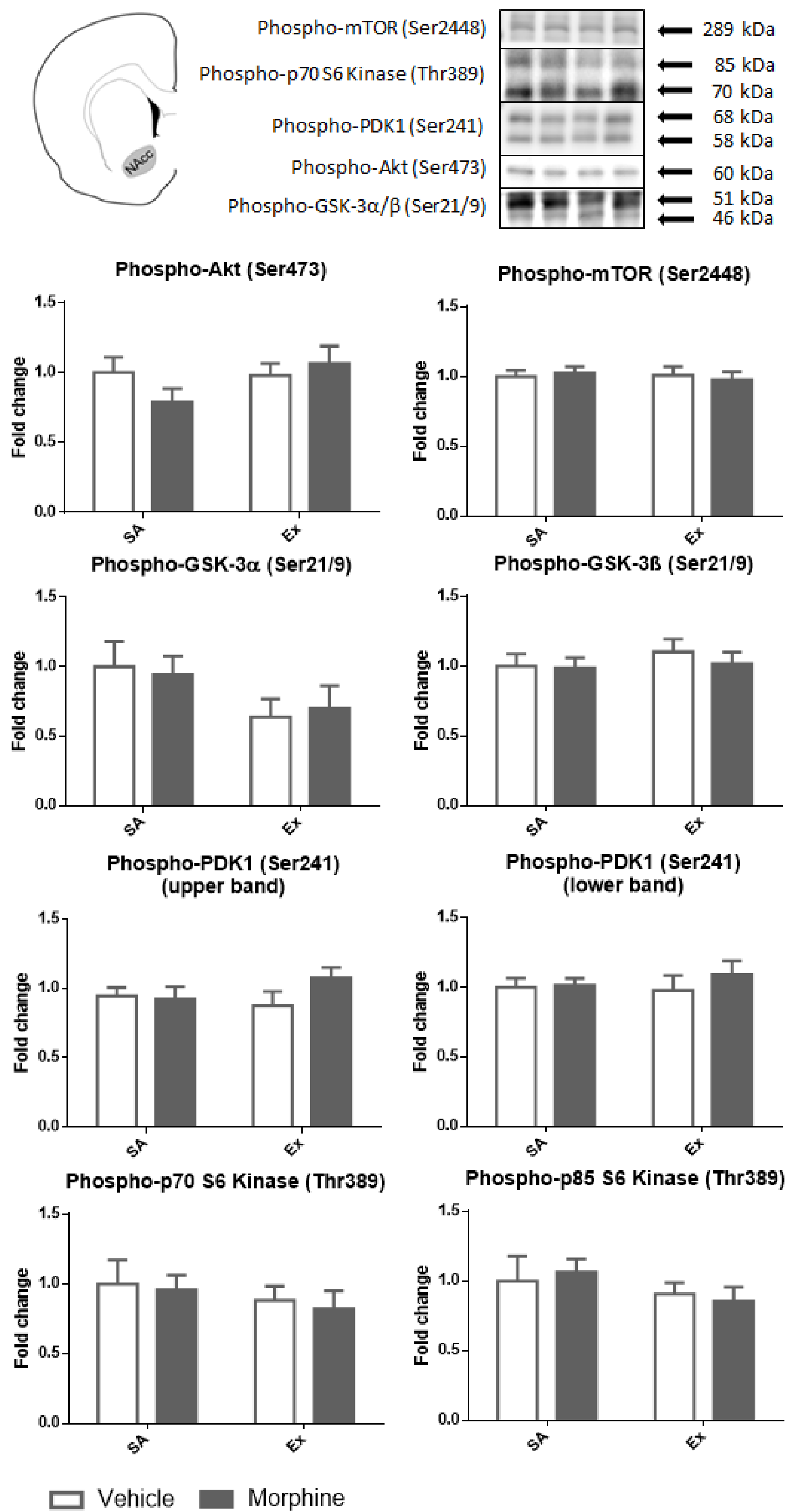


Figure 25: *Representative Western Blots to analyze phosphoproteins in the NAcc, normalizing the data to the total protein in the gel and the mean of the VhSA group (expressed as the mean ± SD).*

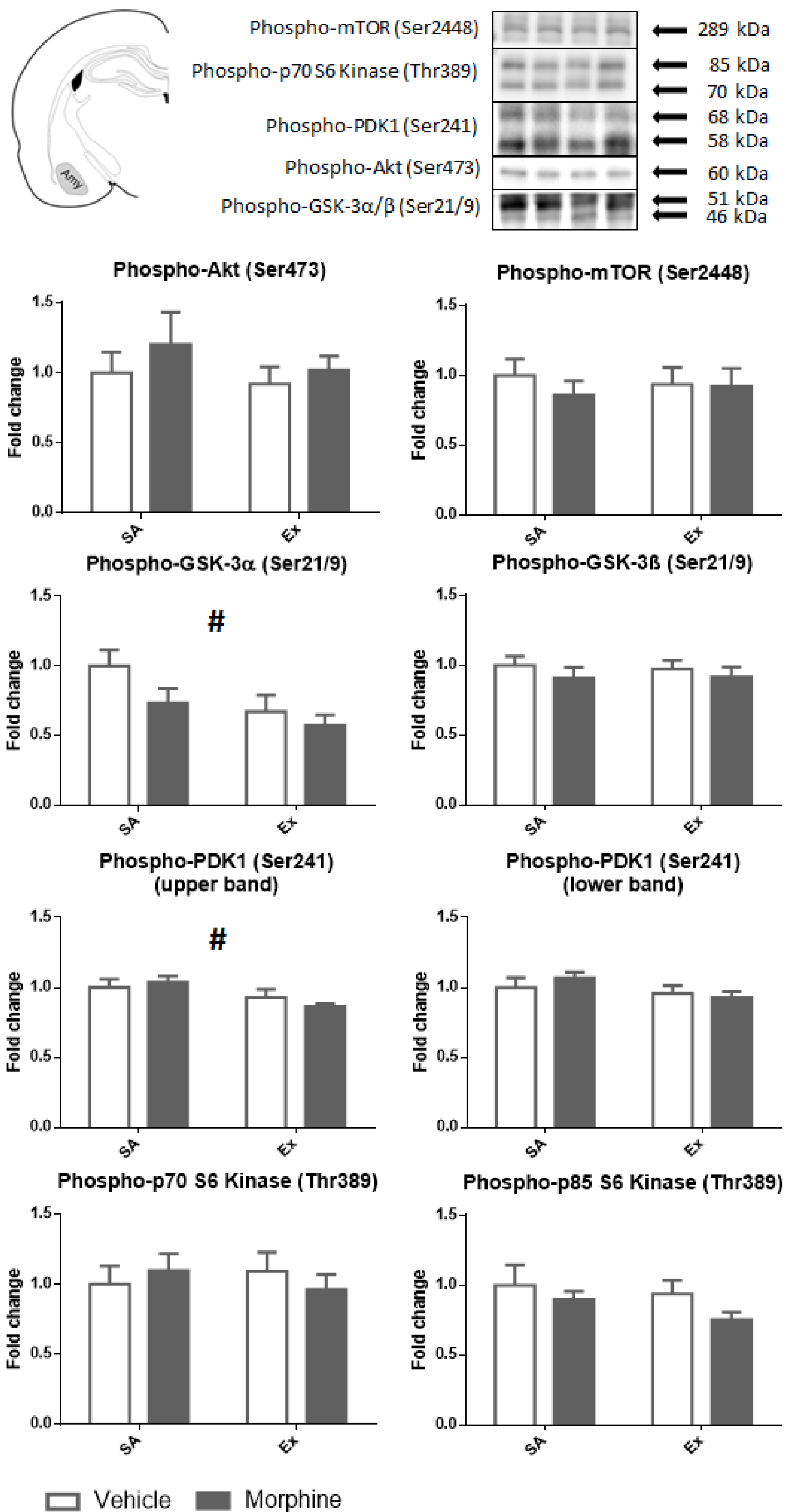


Figure 26: *Representative Western Blots to analyze phosphoproteins in the BLA, normalizing the data to thetotal protein in the gel and the mean of the VhSA group (expressed as the mean ± SD).*

3.3. Discussion

We assessed the effects of morphine self-administration and the subsequent extinction of this behaviour on the expression of several genes and on the levels of specific phosphorylated proteins of the mTOR signalling pathway in three brain areas related to reward learning and extinction: the amygdala, the NAcc and the prefrontal cortex.

The morphine self-administration program employed only affected the expression of the *Rptor* and *Eif4ebp2* genes in the amygdala, an effect that persisted after extinction (Table 8). The *Rptor* gene encodes the regulatory-associated protein of mTOR (Raptor), a protein in the mTORC1, while the product of the Eif4ebp2 gene is one of the downstream effectors of this complex (Shimobayashi and Hall, 2014). Raptor regulates mTOR kinase activity, and it also recruits mTORC1 substrates like the S6 kinases and EIF4E binding proteins like EIF4EBP2 (Hara et al., 2002; Kim and Sabatini, 2004; Ma and Blenis, 2009). The eIF4EBP proteins, in turn, regulate EIF4E activity, which is responsible for the cap-dependent translation of mRNAs (Richter and Sonenberg, 2005). Our dissection of the amygdala mostly included the BLA, an area with an important role in conditioning learning given that it encodes the motivational value of the conditioned stimulus, either appetitive or aversive (Everitt et al., 2003). The BLA also has a role in the formation, retrieval and reconsolidation of drug-related memories (Luo et al., 2013). Indeed, c-Fos activity in the BLA is enhanced in rats showing CPP or conditioned place aversion (CPA) to morphine (Guo et al., 2008). Considering all this evidence together, the enduring increase in mTORC1 activity after morphine self-administration in the BLA (as suggested by the elevated transcription of the *Rptor* and *Eif4ebp2* genes) could contribute to the stabilisation of those morphine-related aversive and appetitive memories that persist even after extinction.

Another interesting result was the variation in *Insr* gene expression that decreases drastically after morphine self-administration relative to rats exposed to the vehicle alone (Table 8). The *Insr* gene encodes the insulin receptor, one of the upstream activators of the PI3K/Akt/mTOR pathway (Niswender et al., 2003; Taha and Klip, 1999). Moreover, morphine can also activate this pathway through μ opioid receptors (Law et al., 2000; Polakiewicz et al., 1998). It is plausible that our results could reflect the opioid inhibition of insulin signalling due to crosstalk between the downstream signalling pathways of both receptors, as shown previously in cell cultures (Li et al., 2003). These results are also consistent with the evidence that a chronic morphine regime downregulates the IRS2-Akt signalling pathway in the ventral tegmental area (Russo et al., 2007). This dampened endogenous insulin signalling might contribute to the development or expression of morphine withdrawal syndrome. Indeed, insulin

administration reduces withdrawal symptoms in rats (Singh et al., 2015). Furthermore, rats that self-administered morphine did not display the decrease over time that vehicle-treated rats did. This increase in the Insr might suggest recovery from withdrawal syndrome although direct evidence for this is lacking.

Previous works in the literature have suggested that SGK1 is up-regulated after opiate exposure. For example, Sgk1 mRNA expression is enhanced in whole brain lysates after chronic oxycodone administration, a μ opioid receptor agonist (Hassan et al., 2009). Elsewhere, Sgk1 mRNA levels and activity was seen to increase in the VTA after 7 days of passive morphine administration (i.p. 15mg/kg: Heller et al., 2015) and chronic morphine administration passively increases mTORC1 activity in the VTA, while decreasing that of mTORC2. Such treatment also decreased the soma size of VTA dopaminergic neurons, an effect that increased cell activity but that decreased dopamine output in the NAcc shell. These effects were blocked by overexpressing Rictor in the VTA, indicating that reduced mTORC2 activity mediates these adaptations (Mazei-Robison et al., 2011). SGK1 activation is mediated by the mTORC2 complex (García-Martínez and Alessi, 2008), and has previously been shown to play an important role in spatial memory consolidation (Lee et al., 2006; Tsai et al., 2002) and LTP (Ma, 2006). In spite of all these data, we only observed a marginal increase of Sgk1 mRNA expression (in all the brain areas studied) that did not reach statistical significance, suggesting a crucial effect for contingency in the effects of opiates on this mTORC2 effector (Table 8).

We also found changes independent of the treatment, but that rather reflected the experimental phase. The Akt1 and Igfr2 genes were more strongly expressed in the amygdala in the groups that underwent extinction training, even in the rats that received a saline solution during the self-administration phase. As opposed to the amygdala, Igfr2 expression in the PFC was reduced in both groups after extinction (Table 8). These changes could reflect the natural regulation of these genes over the lifetime of the rats or maybe, they were a result of the experimental manipulations the rats were subjected to (surgery, handling, behavioural experiments…). Apart from the changes in gene expression, we also found variations in the phosphorylation of GSK-3α (Ser21/9) and of the 68kDa isoform of PDK1 (Ser241), both of which changed after extinction in the two groups irrespective of their prior treatment (Fig. 26). The levels of both phosphoproteins decreased in the BLA after extinction, and those of phospho-GSK-3α (Ser21/9) also tended to fall in the NAcc (Figure 25).

There are some limitations to this study that need to be discussed. Firstly, we lose the registry of inactive lever presses. Although we have the data from the saline self-administering rats that could account to some extent for non-specific lever presses,

we may be overseeing potential effects of morphine self-administration in locomotor activity. The second limitation is that some effects of the previous food-reinforced operant conditioning on mTOR signalling might be affecting our results. This possibility nonetheless seems unlikely because the mTOR pathway is not involved in food reward-seeking (Wang et al., 2010). In spite of these limitations, our findings open the door to new experiments using pharmacological or genetic manipulations of the mTOR pathway in the regions studied here that will provide more definite evidence for the causal involvement of this pathway in the rewarding actions of morphine and the extinction of morphine-related behaviours.

Chapter 4

Study about the effects of heroin self-administration and forced withdrawal in the expression of genes related to the mTOR network

4.1. Methods

4.1.1. Animals

Male rats of the Lewis strain were used (Harlan International Ibérica, n=48), between 300-320g of weight at the beginning of the experiments. The animals were kept in the vivarium in a light-dark cycle (on at 08:00 am), at a constant temperature (20±2°C) and relative humidity (50%±10%), with ad libitum access to water and food (standard commercial rodent diet A04/A03: Panlab). Since their arrival, the animals were housed in groups of three until they were operated a week before the start of the self-administration sessions. Then they were individualised to prevent the rats from biting the catheters of their cage mates. All the animals were maintained and handled according to European Union guidelines for the care of laboratory animals (EU Directive 2010/63/EU governing animal experimentation), and the Ethical Committee of UNED approved all the experimental procedures.

4.1.2. Experimental groups

Animals were randomly assigned to the following groups: Heroin Self-Administration (HSA, n=8), Vehicle Self-administration (VhSA, n=8), Heroin Self-administration Seeking Test (HSAST, n=8), Heroin Withdrawal (HW, n=8), Vehicle Withdrawal (VhW, n=8) and Heroin Withdrawal Seeking Test (HWST, n=8).

4.1.3. Surgery

All the animals were submitted to intravenous catheterisation surgery. Rats were anaesthetized with an isoflurane/oxygen mixture (5% isoflurane during induction; 2% ±0.5% for maintenance), and a polyvinyl chloride catheter (0,16mm i.d.) was inserted into the right jugular vein of the animal approximately at the level of the atrium and secured there with surgical thread. The catheter was fixed subcutaneously around the neck, exiting the skin at the midscapular region. A pedestal of dental cement was then mounted on the skull of the rat to attach the tethering system. After surgery, the rats were allowed to recover for 7 days and an NSAID (meloxicam - Metacam™: 15 drops of a 1.5 g/ml solution per 500 ml of water) was added to the drinking water. Until the end of the self-administration procedure, the catheters were flushed daily with a sterile saline solution containing sodium heparin (100 IU/ml) and gentamicin (1mg/ml) to maintain catheter patency and to prevent infections.

4.1.4. Apparatus

Twelve operant conditioning chambers (l=300mm; w=245mm; h=328mm) (Coulborne Instruments), each equipped with a microliter injection pump, were used for the heroin self-administration and seeking tests. A catheter was connected to the rat and held in place with a spring-tether system, and a rotating swivel, which allowed

the animals to move freely inside the chamber. Two levers placed 14cm apart were available throughout all the sessions, one of them inactive.

4.1.5. Self-administration

A week after recovering from surgery, the rats underwent 10 daily sessions of either heroin or vehicle self-administration. During the light phase of the light cycle, for 6 hours rats were allowed daily access to heroin (0.075 mg/kg in a sterile saline -0.9% NaCl- solution) or its vehicle alone under a fixed-ratio 1 reinforced schedule. The house light was off during the sessions, although we allowed some environmental light to respect the light/dark cycle of the animals. During these sessions, one active lever press resulted in a heroin infusion (100 μL delivered over 5 seconds) and a cue light was switched on for 10 seconds. Each active lever press was followed by a 40-second time-out during which the responses had no effects but were still registered. In the first two self-administration sessions, two sucrose pellets were placed on the active lever to facilitate the acquisition of self-administration behaviour.

4.1.6. Seeking tests

The rats of two of the groups underwent seeking tests one day (HSAST) or one month (HWST) after the last self-administration session. These tests were performed under the same conditions of the self-administration, but the active lever presses were not followed by an infusion, and the duration of the sessions was three hours.

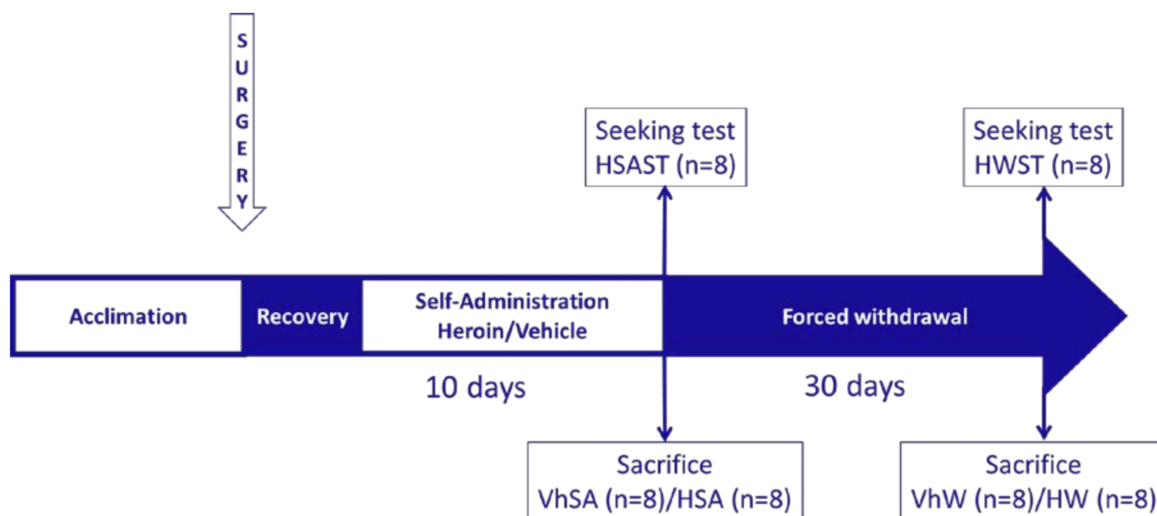


Figure 27: *Experimental schedule for this experiment.*

4.1.7. Tissue collection and processing

The rats of the other groups were weighed and euthanised by decapitation one day (HSS & VhSA) or one month (HW & VhW) after the last self-administration session,

between 11:00 and 13:00 a.m. The rat's brain was extracted and submerged in isopentane chilled on dry ice for ten seconds and stored at -70 ºC.

For the dissection of the BLA, each brain was embedded in TissueTek (Sakura, 4583) and tempered at -20 ºC in a cryostat chamber (Microm, Cryostat HM 500O). After one hour, slices about 300 µm thick were collected and dissected with sterile equipment. The dissection of the BLA was performed following Paxinos and Watson, 2013 (Figura 28), and the tissue was kept in dry ice until they were stored at -70 ºC.

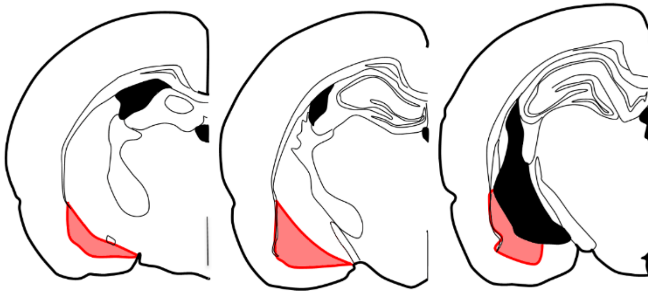


Figure 28: *Graphical representation of the sections of the rat brain with the dissected area highlighted in red.*

Samples were weighed and homogenized with a pellet pestle (Sigma-Aldrich, Z359971) in HEPES buffer (50 mM, pH 7.5, Sigma H3375) prepared in diethylpyrocarbonate (DEPC)-treated water, and containing sucrose (320 mM, Sigma S1888), protease (Roche complete EDTA-free 11.873.580.001) and phosphatase inhibitors (Roche PhosSTOP 04.906.837.001) and sodium butyrate (20 mM, Sigma B5887). The homogenates were kept on ice for 10 min and then centrifuged for 10 min at 1000 g at 4 ºC. The supernatant was separated into three different tubes: an aliquot equivalent to 3-4 mg of tissue (≤80 µL) in a tube with 800 µL of QIAzol (Qiagen 79306) for RNA isolation; another aliquot in a tube with loading buffer for western blotting; and the rest into an empty tube for protein quantification and other experiments. All the aliquots were stored at -70 ºC.

4.1.8. RT-qPCR analysis

The total RNA was extracted and precipitated using the chloroform, isopropanol and ethanol method (Chomczynski and Sacchi, 1987) with glycogen as a carrier. The precipitate was dissolved in RNAse free water, and the concentration and RNA integrity (as indexed by the RIN value) was assessed in a bioanalyzer (Agilent 2100). The RNA concentration in each sample was adjusted by adding RNAse free water and, to avoid genomic DNA contamination, DNAse digestion was performed (DNAse I, Amplification Grade, Invitrogen) following the manufacturer's instructions. Finally, the samples were retrotranscribed using a commercial kit (Biorad iScript™ cDNA Synthesis Kit), and the cDNA was diluted 1:10 in nuclease-free water. PCR assays were performed on a real-time PCR detection system (CFX9600, Biorad) with an SSO Advanced SYBR mix (Biorad) using the primers indicated in the supplementary materials section. We ran

duplicates of all the samples along with a No-Template Control. We discarded the data of any assay with an unusual amplification or melt curve, or if the difference between duplicates was higher than one cycle. The relative expression of each gene calculated as described in Pfaffl, 2001 using Gapdh as a reference gene and the reaction efficiencies were obtained using LinRegPCR software (Ruijter et al., 2009), and normalised respect to the VhSA group.

Table 9: *List of primers pairs used for the RT-qPCR asssays*

Gene	Primer sequences (5'-3')	
	Sense	Antisense
Akt1	CGCTTCTTTGCCAACATCGT	TCATCTTGATCAGGCGGTGT
Akt2	GGCACGCTTTTATGGAGCAG	ATCTCGTACATGACCACGCC
Gapdh	TCCCTGTTCTAGAGACAG	CCACTTTGTCACAAGAGA
Gsk3a	GCCCAACGTGTCCTACATCT	TTGGCGTCCCTAGTACCTTG
Gsk3b	CCGAGGAGAGCCCAATGTTT	CTTCGTCCAAGGATGTGCCT
Igf1r	ATCTCCGGTCTCTAAGGCCA	CCAGGTCTCTGTGGACGAAC
Igf2r	TCACAATCGAGGTGGACTGC	CACCCGGTGACAGACATTGA
Insr	GCTTCTGCCAAGACCTTCAC	TAGGACAGGGTCCCAGACAC
Mtor	GGTGGACGAGCTCTTTGTCA	AGGAGCCCTAACACTCGGAT
Rp6kb1	ACTGGAGCACCTCCATTCAC	GCTTGGACTTCTCCAGCATC
Pdk1	GAAGCAGTTCCTGGACTTCG	GCTTTGGATATACCAACTTTGTACC
Pik3ca	GAGCACAGCCAAGGAAACTC	TCTCCCCAGTACCATTCAGC
Rptor	CTTGGACTTGCTGGGACGAT	ATGAAGACAAGGAGTGGCCG
Rictor	CCGTCGCAGCAATCAAAGAC	CCCCCAATTCGATGAGCCAA
Rps6	CGTCTTGTTACTCCCCGTGT	GCCTACGTCTCTTGGCAATC
Eif4ebp2	TCCTGGCGCCTTAATGGAAG	AAGATGTGGCTGGACAGAGC

4.1.9. Statistical analyses

The data obtained from the self-administration was analysed with a mixed-model repeated-measures approach, and the within-subject correlations were modelled using the first-order autoregressive covariance structure. The analysis had Sessions as within-subjects factor (ten levels) and Treatment (three levels: Heroin_tissue, Heroin_test and Vehicle_tissue) and Withdrawal (Two levels: 1_day, 30_days) as between-subject factors. Even though at that time of sacrifice rats that performed the seeking tests had the same treatment than the rats that self-administered heroin, we wanted to ensure that their behaviour had been similar so far.

Since the incubation of heroin seeking is a well-studied phenomenon (Roura-Martínez et al., 2019; Shalev et al., 2001), we used a one-tailed t-test to asses it (the test

was still significant with a two-tailed test). We calculated Cohen's D as an effect size estimate.

For the analysis of the biochemical variables, we used a two-way ANOVA with two factors: Treatment (Two levels: Heroin and Vehicle) and Withdrawal (Two levels: 1_day and 30_days).

Prior to these tests, we checked for normality and homoscedasticity, and applied log_{10}, square root or reciprocal transformations. If the assumptions were still violated, a Kruskal-Wallis non-parametric test was performed instead of ANOVAs.

Software

The statistical analyses were performed using SPSS 24 (IBM) and InVivoStat (Mockett Media). The graphs were designed using PRISM 6 (Graphpad software Inc.)

4.2. Results

4.2.1. Heroin self-administration

The rats of the groups that self-administered heroin, either the tissue-collection or the test groups, acquired the lever-press behaviour and, as the sessions went on, self-administered more heroin (Figure 29). There was an effect of the Session factor ($F_{9,374}=4.48$, $p<0.0001$), indicating that the behaviour changed throughout the sessions. We also found an effect of the Treatment factor ($F_{2,42}=161.92$, $p<0.0001$), and an Session*Treatment interaction ($F_{18,374}=7.83$; $p<0.0001$) (Figure 29B). The pos-hoc analyses indicated that, as expected, the vehicle-treated animals displayed fewer responses in all the sessions than the groups that self-administered heroin, both in the tissue or the test group. There was a session in which the test group and the tissue groups behaved significantly different, but as there was no overall difference this small difference will not compromise our results.

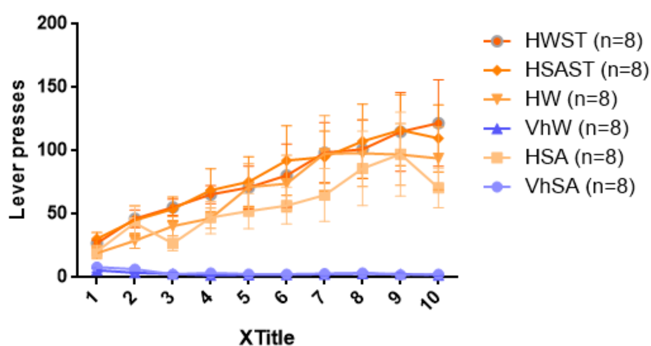
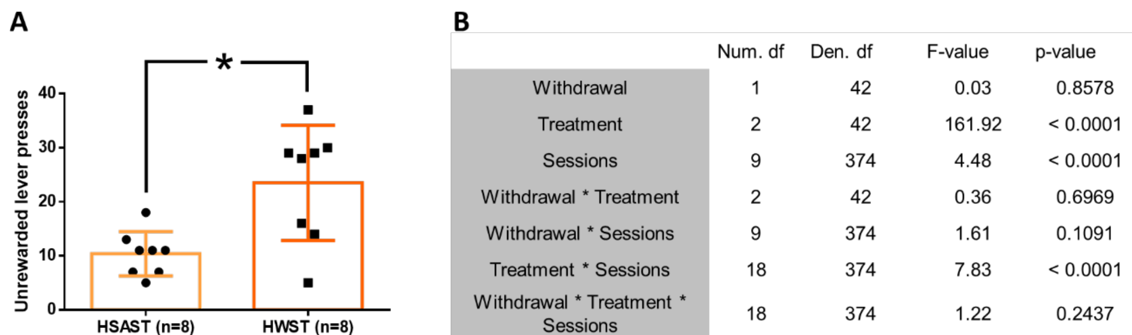


Figure 29: *Graphical representation of the self-administration data (VhSA - Vehicle self-administration; HSA - Heroin self-administration; VhW - Vehicle withdrawal; HW - Heroin withdrawal; HSAST - Heroin self-administration seeking test; HWST – Heroin withdrawal seeking test)*

4.2.2. Incubation of heroin seeking

The drug-seeking incubation phenomenon was evident in the rats of the groups that performed the seeking tests. The rats that performed the test 30 days after the last self-administration session displayed more lever presses than the rats which performed the test one day after the last session (Figure 30A) ($t_{8.022}$=-2.669, p=0.014, d=1.89). This effect was specific of the previously reinforced lever, as we found no differences in the number of inactive lever presses of both groups (t_{14}=-0.460, p=0.653)



	Num. df	Den. df	F-value	p-value
Withdrawal	1	42	0.03	0.8578
Treatment	2	42	161.92	< 0.0001
Sessions	9	374	4.48	< 0.0001
Withdrawal * Treatment	2	42	0.36	0.6969
Withdrawal * Sessions	9	374	1.61	0.1091
Treatment * Sessions	18	374	7.83	< 0.0001
Withdrawal * Treatment * Sessions	18	374	1.22	0.2437

Figure 30: A) *Results of the seeking tests evidencing the incubation of heroin seeking.* ***B)*** *Table of overall tests of model effects for self-administration data.*

4.2.3. RNA integrity

All the samples had a RIN value of 7 or higher and, unless exceptions and the 260nm/280nm absorbance ratios were 1.8 or higher. We ensured that all the samples of the same area had similar *Gapdh* expression Cts (Max. range=2Ct).

4.2.4. Gene expression

We found that the expression of several genes increased in the BLA after heroin self-administration, and these effects were still evident after 30 days of withdrawal. The genes which showed significant Treatment effects in the ANOVA were *Akt2* (F1,28=5.763, p=0.023, η2=0.158), *Gsk3a* (F1,28=5.556, p=0.025, η2=0.162), *Igf1r* (F1,28=4.931, p=0.035, η2=0.147) and *Igf2r* (F1,28=6.277, p=0.018, η2=0.161). Apart from the latter, *Eif4ebp2* expression also seemed to increase, but this surge did not reach the traditional threshold for statistical significance (F1,28=4.004, p=0.055, η2=0.12).

We also found a significant interaction between the Treatment and Withdrawal factors concerning the expression of *Rictor* in the BLA (F1,28=4.293, p=0.021, η2=0.118). The simple effects analysis showed that the animals had higher levels of *Rictor* expression after heroin self-administration compared to their controls (F1,28=5.094, p=0.032, η2=0.182), and also that there was an increase after the

withdrawal in the expression of *Rictor* in the BLA of the rats that self-administered vehicle ($F_{1,28}=7.104$, $p=0.013$, $\eta^2=0.254$), while the rats that self-administered heroin had already elevated levels before withdrawal.

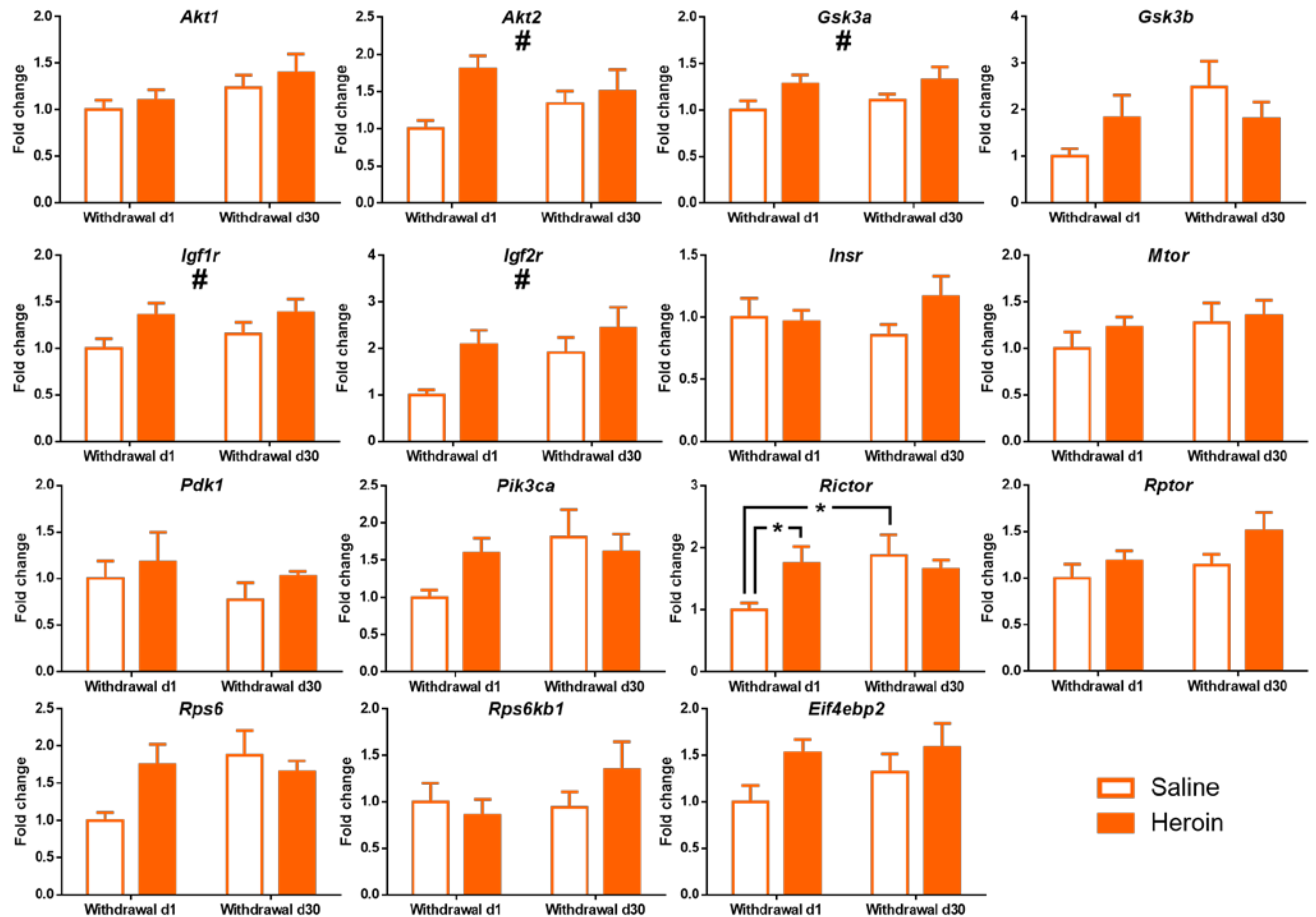


Figure 31: *Mean and standard deviation of the relative expression of the genes studied normalised to Saline/Withdrawal 1 (VhSA) values. # Significant effect of the Treatment factor ($p < 0.05$). *Significant effect of the simple effect analyses ($p < 0.05$).*

4.3. Discussion

In this study we analysed the gene expression of several elements related to the mTOR network in Lewis rats that had extended access to heroin or saline, either one or thirty days after the last self-administration session. We also confirmed in a separate group of rats the existence of incubation of heroin seeking using this behavioural protocol.

Our main finding was an increase in the expression of Akt2, Gsk3a and the receptors Igf1r and Igf2r in the rats that had self-administered heroin compared to their vehicle controls, a change still evident after one month of withdrawal. These genes are closely related to the mTOR network. The activation of Insulin-like growth factor receptors

promotes the phosphorylation of PI3K, which in turn mediates the phosphorylation of Akt (Alessi et al., 1996; Dudek, 1997), and GSK3 inhibition by is regulated by Akt kinase activity (Cross et al., 1995). GSK3 activation in the BLA is increased after exposure to drug-associated cues and is involved in the association of incentive value to these cues (Wu et al., 2011). Although there are no previous reports of overregulation of this pathway in the BLA, there are reports of similar effects in vitro or in other brain areas. For example, plasma levels of IGF1 were transiently increased after intracerebroventricular administration of morphine, another opioid (Hashiguchi et al., 1996). In vitro studies show that opioid stimulation elicits an increase in Akt activation (Li et al., 2003; Polakiewicz et al., 1998), but the studies in vivo show a more complex regulation. Although acute morphine administration produced an increase in Akt activity in the Accumbens (but not in the Striatum), chronic administration had the opposite effect, although total protein levels remained unchanged (Muller and Unterwald, 2004).

Similarly, in other in vivo studies, chronic morphine produced a decrease in Akt and GSK3 activity in the VTA, while PI3K and PDK1 remained unchanged. These changes were related to a decrease in the rewarding properties of morphine and are supposed to be involved in the tolerance to opioids (Mazei-Robison et al., 2011; Russo et al., 2007). Whether our findings in the BLA are related or not to tolerance or any other addiction-related phenomenon will require further testing.

We also found an interesting trend regarding *Eif4ebp2* expression, which tended to be enhanced in the rats that self-administered heroin, even after thirty days of withdrawal. This is interesting because in a previous study, we found a similar effect in rats that had self-administered morphine, and this effect persisted after fifteen extinction sessions (Ucha et al., 2019a). *Eif4ebp2* encodes one of the effectors of the mTORC1 (Shimobayashi and Hall, 2014) which, when not phosphorylated, inhibits cap-dependent mRNA translation by binding to the EIF4E (Richter and Sonenberg, 2005). The activation of EIF4E through mTORC1 is also mediated by Akt (Wendel et al., 2004), which showed elevated expression, as discussed before. In another study, the levels of EIF4EBP (total and phospho-EIf4EBP) were increased in the VTA, but not in the accumbens, after chronic morphine administration (Mazei-Robison et al., 2011). There is evidence as well of opioid-stimulated phosphorylation of EIF4EBP1 and EIF4EBP2 in vitro (Polakiewicz et al., 1998).

We also found a surge in the expression of Rictor, one of the proteins of the mTORC2, in the rats that self-administered heroin compared to their controls. Remarkably, in this case, after the withdrawal, both heroin and vehicle-treated animals had a similar increase in Rictor expression. It is possible that the change in the rats of the control

groups is a result of the experimental manipulations or the natural course of the regulation of this gene. This notwithstanding, the increased levels of Rictor transcripts after heroin self-administration could imply an involvement of the mTORC2 in the changes we have seen in the BLA of heroin treated animals. Apart from the classic PI3K/Akt pathway mentioned before, there are several activators of Akt, and mTORC2 is one of them (Sarbassov et al., 2005). In another study, the changes in opioid-related Akt activation in the VTA were studied by evaluating the phosphorylation sites of both pathways. The authors found that both of them were affected, but the related changes in behaviour and VTA cell physiology were dependent on mTORC2 activity only (Mazei-Robison et al., 2011).

Chapter 5

General discussion and conclusions

5.1. General discussion

This Thesis aimed to study some behavioural and neurochemical factors relevant to substance use disorders. We have examined some of the mechanisms underlying several aspects of addictions ranging from the study of impulsivity, a trait closely related to addiction, to the role of intracellular signalling in opioid self-administration or subsequent extinction or abstinence.

Regarding the study on impulsivity, we have provided the first evidence for a dissociation between the medial and lateral division of the OFC in impulsive action and impulsive choice. We suggest that CB_1 and AMPA receptors in the mOFC are positively coupled to the expression of impulsive choice while $GABA_A$ and AMPA receptors in the lOFC are markers of impulsive action. It would be interesting to conduct some functional studies interfering with or augmenting the expression of these genes to ascertain if there is a causal relationship between the gene transcription variations and the different varieties of waiting impulsivity that we have studied in this work. The use of viral vectors to overexpress or interfere with *Cnr1* expression in targeted neural populations is a promising strategy that we would like to adopt in the near future. It would also be interesting to study if the mTOR network in the OFC is involved in impulsivity. Impulsivity has already been linked to mTOR signalling in the striatum (Lee et al., 2017). Although impulsivity is supposed to be a stable trait along the lifetime of an individual, substance use disorders have also proved to be able to affect impulsivity (Perry and Carroll, 2008). In fact, drug-induced increased impulsivity could be one of the factors behind the elevated risk of relapse. These changes could be mediated by the mTOR network in the OFC or any other area related to impulsivity, and in that case, we could also target the pathway to prevent it (during the writing of this Thesis, cortical mTOR signalling was identified as a key aspect of ethanol-induced impulsivity [Starski et al., 2019]).

In the experiments described in chapters 2 and 3, we have addressed the relationship between the expression of several elements of the mTOR pathway, operant self-administration of morphine or heroin under extended access, and subsequent extinction or forced withdrawal associated to incubated drug-seeking. Of the three areas studied in the study with morphine, the most interesting results were found in the amygdala. The role of this area in the processes of drug addiction and relapse is well known (Kilts, 2001; See et al., 2006) but, to our knowledge, no one has previously observed the potential involvement of the mTOR pathway in this limbic structure. The genes and phosphoproteins identified are mainly involved in regulating protein synthesis, and they may also be recruited during memory formation and

reconsolidation and are known to be related to opioid-induced changes in behaviour or neurophysiology in other brain areas, concurring with earlier data.

The absence of results in the other areas led us to focus on the BLA in the last experiment with heroin. In this study, we have found changes at the transcriptional level in several genes of the mTOR pathway in the BLA caused by heroin self-administration. Surprisingly, we did not find any common effect of the self-administration of morphine and heroin nor did we find any relevant effect related to the incubation of drug-seeking with this protocol. It is possible that the BLA is not a key piece in the mTOR regulation of the incubation of drug-seeking, and we should be focusing on other areas like the central nucleus of the amygdala, as suggested in a recent study by our group (Roura-Martínez et al., 2019).

The main limitation of these studies is the fact that gene expression changes are not necessarily associated with changes in protein levels. Moreover, even if they were, most of the proteins of the mTOR network have to be phosphorylated at certain sites for the changes to be functional. Apart from this, this work should be read bearing in mind that the animal models used here, although helpful when trying to understand isolated traits related to substance use disorders, are incapable to comprehend the complex phenomena involved in human addictions. Having acknowledged these limitations, we think that our results add to the growing evidence of the implication of the mTOR network in opioid addiction, specifically in the BLA, and it would be interesting to study more directly the therapeutic value of this signalling pathway in opioid-related disorders.

5.2. Conclusions

- Premature responding is associated with lower gene expression levels of the GluA1 AMPA receptor subunit and the α1 $GABA_A$ receptor subunit in the lateral, but not medial orbitofrontal cortex.
- Delay aversion is associated with higher gene expression levels of CB_1 receptor and the GluA1 AMPA receptor subunit in the medial but not in the lateral orbitofrontal cortex.
- Extended-access to morphine self-administration elicits an increase in *Raptor* and *Eif4ebp2* expression which are still evident after extinction.
- *Insr* expression was decreased after vehicle self-administration while the opposite effect was observed in the rats that self-administered morphine.
- Extended access to heroin self-administration elicits an increase in *Akt2*, *Gsk3a*, *Igf1r* and *Igf2r* expression, which was still evident after forced withdrawal in a protocol that induced the incubation of heroin seeking.

- *Rictor* expression increased after vehicle self-administration and subsequent withdrawal; however, the rats that self-administered heroin already had elevated expression levels of *Rictor* from the first day of withdrawal.

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