Study of molecular targets and neurobiological mechanisms involved in compulsive overeating

Ana María Gallego Román

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Thesis director: Prof. Rafael Maldonado
PhD. Miquel Martín
PhD. Elena Martín

Departament de Ciències Experimentals i de la Salut

Universitat Pompeu Fabra Barcelona

A mi madre, la eterna luchadora.
Quien me inculcó que aquello en lo que creemos debemos demostrarlo más con nuestros actos y menos con nuestras palabras.

A Ivan, mi compañero de vida y apoyo incondicional.
Gracias por ayudarme a reconocer que lo mejor de la vida está en los detalles más simples.

ABSTRACT

Binge-eating disorder (BED) is a chronic eating disturbance that affects 1.6-2.0% of people worldwide. It is characterised by recurrent episodes of consuming large amounts of food in a short period, together with a sense of loss of control without using compensatory measures. This disorder is associated with significant psychiatric comorbidity and other eating disturbances like obesity and food addiction. Thus, BED is a complex multifactorial disorder whose neurobiological mechanisms involved are still unknown. The present Thesis characterised the involvement of a specific neuronal subpopulation in the nucleus accumbens expressing the dopamine receptor type-2 in binge-like eating, by comparing different dietary conditions and using a cell-specific transcriptomic approach. The bioinformatic analysis demonstrated similarities among different disorders, such as drug addiction and memory and learning task, which suggested that repetitive episodes of compulsive overeating may produce changes in dopamine signalling that differ from those occurring in obesogenic conditions. Specific pharmacological and adenoviral gene delivery approaches provided insights into understanding the neurobiological mechanisms involved in BED, which could help identify new pharmacological approaches to address this pathology. In addition, a well-characterised rodent model that recapitulates most of the symptoms described in psychiatric manuals for BED was proposed as a preclinical tool to explore the aetiological or susceptible factors to suffer this disorder in rodents without comparing to other eating disturbances.

RESUMEN

El trastorno por atracón (TA) es un desorden alimentario crónico que afecta al 1,6-2,0% de las personas en todo el mundo. Se caracteriza por episodios recurrentes de consumo de grandes cantidades de comida en un periodo corto, junto con una sensación de pérdida de control sin utilizar medidas compensatorias. Este trastorno se asocia a una importante comorbilidad psiquiátrica y a otras alteraciones alimentarias como la obesidad y la adicción a la comida. Así pues, el TA es un complejo trastorno multifactorial cuyos mecanismos neurobiológicos implicados son aún desconocidos. La presente Tesis caracterizó la participación de una subpoblación neuronal específica en el núcleo accumbens que expresa el receptor de dopamina tipo 2 en la regulación de la alimentación compulsiva, comparando diferentes condiciones dietéticas y utilizando un enfoque transcriptómico específico de tipo celular. El análisis bioinformático demostró similitudes entre distintos trastornos patológicos, tales como la adicción a las drogas y en tareas de aprendizaje y memoria, lo que sugirió que los episodios repetitivos de sobrealimentación compulsiva pueden producir cambios en la señalización de la dopamina diferentes a los que se producen en condiciones obesogénicas. Los enfoques farmacológicos y de introducción de genes mediante vectores adenovirales específicos proporcionaron información para comprender los mecanismos neurobiológicos implicados en el TA, lo que podría ayudar a identificar nuevos enfoques farmacológicos para abordar esta patología. Además, se propuso un modelo de roedor bien caracterizado que recapitula la mayoría de los síntomas descritos en los manuales psiquiátricos para el TA como herramienta preclínica para explorar los factores etiológicos o susceptibles de padecer este trastorno en roedores sin compararlo con otros trastornos alimentarios

ABREVIACIONES

2-AG	2-arachidonoylglycerol
AMPA	α-amino-3-hydroxyl-5-methyl-4-isoxazeloproprionic acid
ATP	adenosine triphosphate
A2AR	Adenosine A2A receptor
AAV	Adeno-associated virus
AEA	N-arachidonoylethanolamide
AgRP	Agouti-related peptide
BDNF	Brain-derived neurotrophic factor
BE	Binge-like eating
BED	Binge-eating disorder
BLA	Basolateral amygdala
BMI	Body mass index
cAMP	Cyclic adenosine monophosphate
CART	Cocaine- and amphetamine-regulated transcript
CB1R	Cannabinoid type-1 receptor
CB2R	Cannabinoid type-2 receptor
COMT	Catechol-o-methyl-transferase
D1R	Dopamine type-1 receptor
D2R	Dopamine type-2 receptor
DA	Dopamine
DARPP	DA and cAMP-regulated phosphoprotein
DAT	Dopamine transporter
dl	Dorsolateral
dm	Dorsomedial
DSM	Diagnostic and statistical manual of mental disorders
eCB	Endocannabinoid
FC	Free-choice

FR	Fixed ratio
GPCRs	G protein-coupled receptors
Gpe	Globus pallidus external part
Gpi	Globus pallidus internal part
GWAS	Genome-wide association study
IL	Infralimbic cortex
i.p	Intraperitoneal
IP3	Inositol triphosphate
K+	Potassium
L	Layer mAChRs Muscarinic acetylcholine receptors
mGluRs	Metabotropic glutamate receptors
miRNA	microRNAs
MSN	Medium spiny neuron
Na+	Sodium
NAc	Nucleus accumbens
NMDA	N-methyl-D-aspartate
PFC	Prefrontal cortex
PKA	Protein kinase A
PL	Prelimbic cortex
PLC	Phospholipase C
POMC	Pro-opiomelanocortin
PR	Progressive ratio
rTMS	Repetitive transcranial magnetic stimulation
s.c	Subcutaneous
SNpr	Substantia nigra pars reticulada
tDCS	Transcranial direct current stimulation
VP	Ventral pallidum

vm	Ventromedial
VTA	Ventral tegmental area
WT	Wild type
YFAS	Yale food addiction scale

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INTRODUCTION

1 Eating disorders and obesity

1.1 Eating disorders

Eating disorders are defined as persistent disturbances of eating or eating-related behaviour resulting in altered food consumption that significantly impairs physical health or psychosocial functioning (American Psychiatric Association, 2013). Anorexia nervosa, bulimia nervosa and binge-eating disorder are the most well-known eating disorders, although other disorders have been included in psychiatric manuals. These disorders affect millions of people worldwide (around 4.6% of the population), regardless of race, age, nationality, or gender, but they are most common in late adolescents and young women (Mishra, Anand and Umesh, 2017; Zipfel, Schmidt and Giel, 2022).

Such mental disorders are typically chronic, episodic, and often associated with psychiatric comorbidity (such as mood and anxiety, obsessive-compulsive and substance of abuse disorders (American Psychiatric Association, 2013)) and medical sequelae, resulting in significant personal, family, and social costs. However, it is still a common misconception that eating disorders are a lifestyle choice. On the contrary, they are serious and often fatal illnesses that involve severe disturbances in eating behaviour and related thoughts and feelings, with an increasing risk of mortality in the last decades (van Hoeken and Hoek, 2020). This fact, together with the high comorbidity with other psychiatric disorders, have increased concern in the general population and sparked the interest in understanding the underlying neurobiological mechanisms to develop

more accurate approaches to treat these pathologies (Smink, van Hoeken and Hoek, 2012; Schaumberg et al., 2017).

Our current understanding of eating disorders aetiology is based on family, twin and adoption studies that have robustly shown that eating disorders reflect the pattern of complex trait inheritance being influenced by both genetic and environmental factors (Yilmaz, HArdaway and Bulik, 2015). There is evidence that genes and heritability play a role in putting some people at increased risk for an eating disorder, but they can also affect people who do not have a family history related to eating disturbances. Thus, epigenetic mechanisms provide an additional layer of gene regulation that links external and internal environmental stimuli, as well as non-coding genetic variations with transcriptional consequences that alter downstream phenotypes (Hübel, Sarah J. Marzi, et al., 2019).

From a diagnostic perspective, the main psychiatry manual used to diagnose mental disorders by the American Psychiatric Association is the Diagnostic and Statistical Manual of Mental Disorders (DSM). In the fifth version of the manual (DSM-5), the eating disorders section is called "Feeding and Eating Disorders" and the diagnostic criteria are provided for three feeding disorders: pica, rumination and avoidant/restrictive food intake disorders. Eating disorders can be categorized into 3 different main types: **anorexia nervosa, bulimia nervosa** and **binge-eating disorder**.

Both anorexia nervosa and bulimia nervosa have similar symptoms based on distorted body image and self-perception weight or shape. However, these two disorders have essential differences in food-related behaviours. **Anorexia nervosa** is characterized by severely reductions in food intake to lose weight, intense fear of gaining weight or becoming fat, or persistent behaviour that interferes with weight gain. On

the other hand, patients who suffer **bulimia nervosa** show recurrent episodes of binge eating, ingesting an excessive amount of food in a short period, followed by purging or use other methods to prevent weight gain such as self-induced vomiting, diuretics use, fasting or excessive exercise.

According to the DSM-5, some eating disorders can be manifested in ways that resemble addiction disorders, such as **craving and compulsive use**. These similarities may reflect the involvement of similar neurobiological systems, including those involved in regulatory self-control and reward. Therefore, in the highly obesogenic environment in which our Western society finds itself, much attention has been paid to eating disorders characterized by compulsive behaviours and overeating, such as **binge-eating disorder**.

1.1.1 Binge-eating disorder

Binge-eating disorder (BED) is a severe, life-threatening, but treatable eating disorder. It is defined as recurrent episodes characterized by consuming an abnormally large amount of food in short periods of time and experiencing a loss of control over eating during the episodes, without compensatory behaviour such as self-induced vomiting or excessive exercise. Although the binge-eating disorder is closely linked to obesity (Smink, van Hoeken and Hoek, 2012), with up to 50% of people with the binge-eating disorder also suffering from overweight or obesity, people with the binge-eating disorder show a higher risk of developing heart disease, stroke, type 2 diabetes, and cancer compared with people on the same overweight or obesity range without the binge-eating disorder (Kessler et al., 2013; Raevuori et al., 2015; Mitchell, 2016).

1.1.1.1 Prevalence and diagnosis

Prevalence

Epidemiological studies report that 1.6-2.0% of people worldwide suffer from this disorder, being the most common eating disorder in the United States (US) (Gibson-Smith et al., 2015; Cossrow et al., 2016)with around 2.8 million affected people, three times more common than people diagnosed with anorexia and bulimia together. This fact makes this eating disorder the most extended in terms of age, race, gender (1.6% women and 0.8% men) and income levels. In addition, instead of the high comorbidity with obesity (Wonderlich et al., 2009), this disorder has been diagnosed in normo-weight, overweight and obese individuals, revealing independence with the degree of obesity (Wonderlich et al., 2009; Marcus and Wildes, 2012).

Diagnosis

The diagnosis features of binge-eating disorder in the DSM-5 are divided into five main criteria: A, B, C, D and E:

Binge-eating disorder is characterized by recurrent *episodes of binge eating* (**Criterion A**) that are defined as eating in a discrete period of time (within any 2 h period) a larger amount of food than most individuals would eat in a similar period of time (**Criterion A1**). However, to be considered an episode of binge eating, an occurrence of excessive food consumption must be accompanied by a sense of lack of control (**Criterion A2**). Furthermore, the episodes are associated with three (or more) of the eating behavioural features (**Criterion B**): (I) eating more rapidly than usual; (II) eating until feeling uncomfortably full; (III) eating large amounts of food when not feeling physically hungry; (IV) eating alone because of feeling embarrassed and; (V) feeling disgusted with oneself, depressed, or

very guilty afterward. The episodes are, in turn strongly associated with marked distress (**Criterion C**) and must be recurrent, on average, at least once per week for 3 months (**Criterion D**), classifying the severity of this disorder in: I) Mild (1-3 binge-eating episodes per week); II) Moderate (4-7 binge-eating episodes per week); III) Severe (8-13 binge-eating episodes per week); and IV) Extreme (14 or more binge-eating episodes per week). Notably, the binge eating is not associated with the recurrent use of inappropriate compensatory behavior as in bulimia nervosa and does not occur exclusively during the course of bulimia nervosa or anorexia nervosa (**Criterion E**). In addition, the type of food consumed during binges varies across subjects and episodes. Thus, binge eating disorder appears to be characterized more by an abnormality in the amount of food consumed than by craving for a specific nutrient (American Psychiatric Association, 2013).

1.1.2 Palatable food as “addictive” substance

Despite not having a specific food that is always binge eaten during the episodes, certain types of foods with increased content in sugar and fat are mainly craved. In the recent year, a body of literature has demonstrated that foods with added fats and refined carbohydrates generate neurobiological and behavioural changes similar to the reported for substance of abuse (Ifland et al., 2015; Markus et al., 2017; Pursey, Davis and Burrows, 2017; Schulte, Potenza and Gearhardt, 2017). Among these alterations are the intense desire for consuming, the increased impulsivity and the loss of self-control over food intake despite the adverse health effects. These behaviours are crucial traits already reported for addictive disorders, revealing an important overlapping

between substances of abuse and the consumption of a certain type of food that can activate the brain's reward system beyond normal conditions as natural reinforcer (Smith and Robbins, 2013; Volkow, Wise and Baler, 2017; Lindgren et al., 2018). However, whether these "addictive" properties are a consequence of addiction-related behaviour or if specific food components could have intrinsic addictive properties similar to drugs of abuse is still under debate (Westwater, Fletcher and Ziauddeen, 2016; Gordon et al., 2018). Independently of the reason that would explain the addictive properties of high caloric and palatable food, they modify in eating-related behaviours in a pathological manner causing very different issues or disorder manifestations that involve metabolic and mental diseases that implicate overeating (Fletcher and Kenny, 2018). In this sense, the principal eating disturbances include binge-eating disorder, food addiction and obesity.

1.1.3 Overlapping and differences between binge-eating disorder and food addiction

Probably, the most difficult to differentiate between them would be binge-eating disorder and food addiction. The concept of food addiction is still controversial and its recognition as mental disorder is not included in the last version of the DSM. Instead, a validated tool, the Yale Food Addiction Scale (YFAS) is widely accepted among the scientific community (Schulte and Gearhardt, 2017). This validated measure to operationalize addictive-like eating behavior applies the diagnostic criteria for substance dependence to the consumption of highly palatable foods. Elevated scores on the YFAS are associated with obesity, binge eating, impulsivity, craving, attentional biases for food cues, adverse bariatric surgery outcomes, and

medical conditions (Meule and Gearhardt, 2014; Pursey et al., 2014). Addictive-like eating based on the YFAS has also been related to patterns of neural response implicated in substance use disorders (Gearhardt et al., 2011), differential response to a dopamine agonist (Davis et al., 2014) and higher loadings on the genetic dopamine multilocus profile (Davis et al., 2013). Thus, the application of addiction-related diagnostic criteria to eating behaviour appears to provide a helpful assessment strategy for investigating the validity of the food addiction concept.

Although these two mental disturbances share multiple transdiagnostic constructs and have a high comorbidity, they can be identified independently. For instance, clinical studies found that at least 50% of obese adults with the binge-eating disorder also met the criteria for food addiction (Davis et al., 2011; Gearhardt et al., 2012). However, not all the individuals who met the food addiction criteria (30% obese) are clinically diagnosed as binge eaters (Davis et al., 2011). The absence of binge-eating disorder in the presence of food addiction demonstrate that they are two independent disorders, but there are two hypotheses that explain this fact.

On the one hand, some authors support that food addiction could be developed after an extensive, severe and compulsive form of binge eating disorder, being food addiction a more acute and pathologically-dense form of binge-eating disorder (Davis, 2013; Gordon et al., 2018). According to this hypothesis, it is postulated that at the beginning there is an occasional overeating that displays no behavioural pathology nor psychiatric disturbances. Later, mild and intermittent “loss of control” eating appears, manifesting as episodic binges that tend to become more compulsive and frequent in some individuals over time.

When these behavioural alterations become severe, a diagnosis of binge-eating disorder may be warranted. This explanation suggests that chronic binge-eating disorder develops into a more severe syndrome showing significant psychopathology and strong addictive tendencies related to food. At this point, it seems more appropriate to describe such conditions as food addiction (**Figure 1**) (Davis et al., 2013).

Figure 1. Schematic representation of ascending dimension reflecting increasing severity and compulsivity, from occasional overeating toward food addiction. Modified from Davis, 2013.

Although this hypothesis would be an explanation, there are substantial differences between both disturbances that have not been contemplated. Binge-eating disorder is an eating-related disturbance that is not always associated with obesity development as is usually observed in food addicted patients. On the contrary, the severity of the binge-eating disorder is strongly linked to other psychiatric comorbidities involving behavioural disturbances such as anxiety, depression and obsessive-compulsive disorder, among others (American Psychiatric Association, 2013). Thus, this eating disorder is not only an abnormal eating-related behaviour but a compulsive-type behaviour associated to food intake largely triggered by negative emotional states or comorbidity with other mental disorders such as obsessive-compulsive disorder and depression (American Psychiatric Association, 2013).

On the other hand, food addiction is closely linked to obesity and the behavioural alterations related to food consumption does not necessary include binge eating episodes. Similar to substance-addicted, individuals can consume abnormal amounts of addictive substance throughout the day by binging or "grazing" (Davis, 2017). This last abnormal form of consumption is described in addictive-like eating where food consumption is characterized by repeated eating (more than twice) of small amounts of food in an unplanned manner without the ability to resist such repeated snacking. This aberrant relationship with food is also described in some obese patients who are unable to stop snacking during routine weight loss therapy (Conceição et al., 2014), which also demonstrates that obesity is not always an isolated metabolic disease. Large amounts of food can be consumed in this way and is one of the principal criteria described in the YFAS scale. However, this feeding pattern is not included in patients that suffer from binge-eating disorder. Thus, another non-linear explanation is proposed (**Figure 2**).

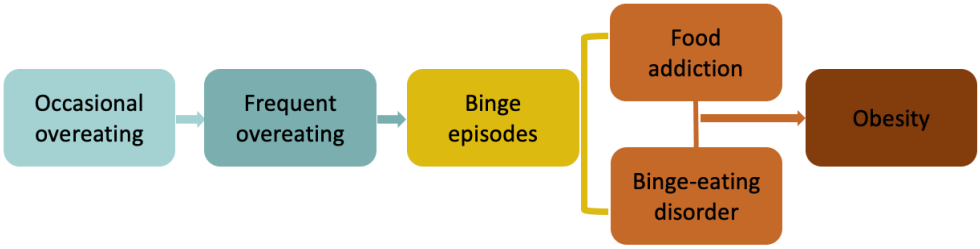


Figure 2. Schematic representation of the ascending dimension reflecting increasing severity and compulsivity in a non-linear way from occasional overeating toward food addiction and binge-eating disorder, causing possible obesity.

1.2 Obesity

Overweight and obesity are defined as abnormal or excessive fat accumulation representing a health risk. This metabolic disorder has reached epidemic proportions, with over 4 million people dying each year due to being overweight or obese (WHO, 2017). Obesity has profound medical consequences and is associated with an increased risk of cardiovascular pathologies, diabetes, cancer, and other diseases (Bray, 2004). The body mass index (BMI) is the current tool used to classify overweight and obesity in adults, calculated by dividing a person's weight in kilograms by the square of their height in meters (kg/m^2). BMI is closely related to obesity and obesity-related comorbidities (González-Muniesa et al., 2017), classifying in: BMI <18.5 - underweight; BMI 18.5-24.9 - average weight; BMI 25-29.9 - overweight; and BMI >30 - obese (WHO, 2017).

Obesity is one side of the double burden of malnutrition, and today more people are obese than underweight in every region except sub-Saharan Africa and Asia (WHO, 2021). Once considered a problem only in high-income countries, overweight and obesity are increasing dramatically in low- and middle-income countries, especially in urban areas. The vast majority of overweight or obese children live in developing countries, where the rate of increase is more than 30% higher than in developed countries. The overweight and obesity prevalence has increased dramatically, affecting more than 1.9 billion adults representing 39% of the total population (39% of men and 40% of women). Of these, over 650 million adults were obese in 2016, representing 13% of the world's adult population (11% of men and 15% of women) (WHO, 2017). Significantly, the prevalence of overweight and obesity in children and

adolescents aged between 5 and 19 has increased radically from 4% in 1975 to over 18% in 2016. This high prevalence results in global health costs are equivalent to 2.8% of the world's gross domestic product, or approximately US$2 trillion (WHO, 2017).

Despite its increased prevalence, the knowledge about this disease is still poor. For instance, specific obese phenotypes are characterized by an excessive desire for food, compulsive consumption, and the inability to restrain oneself despite the desire to eat. These symptoms are similar to those of individuals addicted to drugs as is described in the DSM for substance use disorders (Volkow and O'Brien, 2007). In addition, several patients suffering from obesity are unable to lose weight despite the significant advances in metabolic therapies and treatment of medical complications of obesity. Thus, the prevalence rate of obesity continues to increase, and there are still no effective treatments.

Preclinical and clinical studies have provided multiple lines of evidence about similar neurobiological substrates that involve the same dopamine (DA)-modulated circuits impairments between obese and drug-dependent individuals (Volkow et al., 2011; Tomasi and Volkow, 2013; Volkow and Baler, 2015). These similar neuroadaptations in the reward system consisted in decreased levels of dopamine type-2 receptors activity (Volkow, Wang, Telang, et al., 2008a), as well as a lack of the normal increase in striatal DA releases during the consumption of the reinforcer (drugs or food) (Gene Jack Wang et al., 2014) in both drug-addicted and obese compared to non-addicted and normo-weight, respectively. This attenuated DA response to the reinforcer in the brain's reward pathway generates a mismatch between the experiences of the expected and the

actual reinforcer that may lead to compulsive use or intake of the desired substance (Volkow, Wang, Telang, et al., 2008a; Volkow et al., 2011). Therefore, considering all these observations, it is crucial to consider that some forms of obesity could not be diagnosed exclusively as a metabolic disorder and must be considered the possible comorbidity with a mental disorder (Volkow and O'Brien, 2007; Lerma-Cabrera, Carvajal and Lopez-Legarrea, 2016). Despite several authors arguing to include a component of mental disorder in obesity due to its addictive dimensionality, obesity is not currently included in the DSM-5, neither in the feeding and eating disorders nor in the substance use disorders sections. In this framework, certain foods, mainly highly palatable foods and caloric, with addictive-like properties can promote excessive consumption and increase the risk of developing obesity and eating disorders.

Albeit obesity and binge-eating disorder are different diseases, they are frequently comorbid (Fairburn et al., 2000; Annagür, 2011). Beyond the different eating-related behavioural traits described above, and the absence of obesity in patients diagnosed with binge-eating disorder (Walsh and Boudreau, 2003), objective evidence distinguishes them. Laboratory studies comparing weight-matched obese individuals with and without binge-eating disorder reported that those with binge-eating disorder consume more calories and have a greater functional impairment associated with self-control and decision making (Duchesne et al., 2010; Svaldi, Brand and Tuschen-Caffier, 2010; Balodis et al., 2013), as well as lower quality of life, more subjective distress, and more significant psychiatric comorbidity (linked to behavioural disorders) (American Psychiatric Association, 2013). In addition, contrary to the DA hypo-functionality reported for obese, patients suffering from binge-eating

disorder reveal a possible hypersensitivity to rewards (Davis et al., 2009) that could explain the compulsive overeating reported as principal diagnostic criteria. These differences have been supportered by genotyping studies that reported greater density of D_2R and higher D2 binding potential than obese without the binge-eating disorder (Davis et al., 2008, 2009, 2012). Taken together, obesity and binge-eating disorder have a high comorbidity, but they are independent and are distinguished by their eating behavior and neurobiological characteristics. However, further research is needed to fully unravel the neuropathophysiological mechanisms underlying maladaptive eating behaviors.

2 Neurobiology of eating disorders

2.1 Food intake control

Together with drinking and sex, eating is a natural stimulus with intrinsically reinforcing properties that activate the reward system (Volkow, Wise and Baler, 2017). The regulation of food intake involves close relationships between two main mechanisms: **homeostatic factors** (see next section) or need to eat for energy regulation (balance between energy intake and expenditure), and "**non-homeostatic, allostatic"** or **hedonic factors** (see section 2.1.2) related to the pleasure to eat (Onaolapo and Onaolapo, 2018). In homeostatic feeding, the brain monitors available energy supplies by integrating peripheral signals that usually lead to a state of satiety. The role of allostatic or hedonic feeding was originally to trigger food intake in advance of periods when food was not available. This regulation can outpace energy needs and favour the

perception of rewarding's effects of the food to promote the energy accumulation.

Homeostatic and allostatic systems are activated in all feeding situations to keep energy stores, weight balanced, and nutritional status adapted to the individual environment (**Figure 3**). However, the degree to which each process is activated may depend on the type of food and the subject's physiological state (Rossi and Stuber, 2018). Palatable foods rich in sugar, carbohydrates, fat or even salt are massively craved not only to satiate hunger but also for pleasure (Alonso et al., 2015), prevailing reward-related signals over homeostatic signals that potentiate excessive food intake above the body's energy requirement and leading to eating disorders (Pandit et al., 2011; Caron and Richard, 2017).

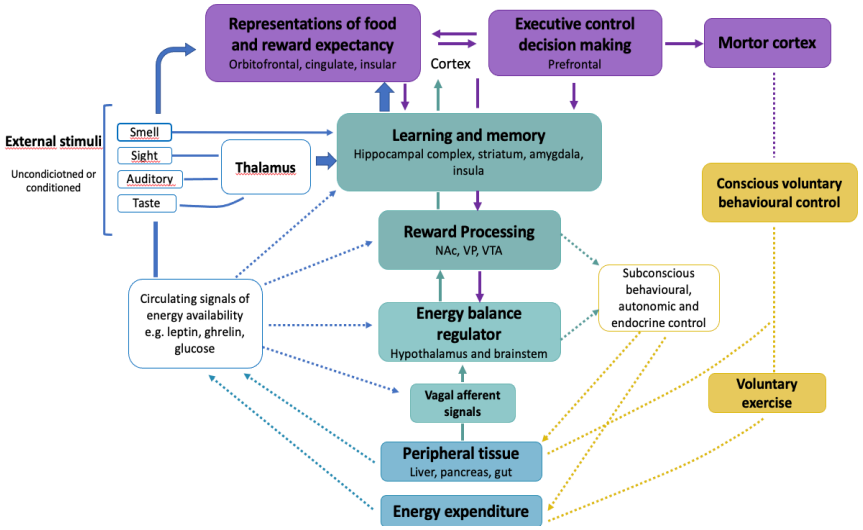


Figure 3. Schematic diagram showing the multiple neural systems and pathways controlling food intake, energy expenditure, and energy balance, with emphasis on interactions between "metabolic," "cognitive," and "rewarding" brain systems. Adapted from Lenard and Berthoud, 2008.

2.1.1 Homeostatic regulation of food intake

Several organs in the body, including the gastrointestinal tract, the adipose tissue, and the central nervous system (CNS), act together to maintain an appropriate energy balance (Abdalla, 2017) through **short-term** and **long-term** regulating processes.

After ingesting a meal, the gastrointestinal tract, containing chemo- and mechano- receptors, sends afferent signals via the vagus nerve to the nucleus of the solitary tract about food information (amount of food and nutrients content, among others). Such information provides "satiety signals" by *short-term signals* determining the meal's beginning and end. After digestion, when nutrients have been metabolized in peripheral tissues or have directly crossed the blood-brain barrier, *long-term signals* are sent to the nucleus arcuatus of the hypothalamus. At the same time, many digestive products and components responsible for their metabolism are integrated and brought together in the brain to inform the metabolic state resulting from food intake (Hopkins, 2016). Following the processing of this information, efferent signals are sent from the brain to affect energy intake and expenditure (Sandoval, Cota and Seeley, 2008).

2.1.1.1 Peripheral regulation

Peripheral control of appetite is regulated primarily by **peptide neurotransmitters** and **hormones** released by the gastrointestinal tract, pancreas, liver, muscle and adipose tissue to provide information about the body's energy status to the CNS (Mishra, Anand and Umesh, 2017). They are generally classified as **orexigenic** and **anorexigenic substances** (summarized in **table 1**).

Table 1. Orexigenic and anorexigenic substances release from peripheral organs. Adapted from Mishra, Anand and Umesh, 2017.

Orexigenic substances	Anorexigenic substances
Neuropeptide Y	α-MSH
Agouti-Related Protein (AgRP)	Leptin
Melatonin concentrating hormone	Serotonin
Orexin A	Corticotropin releasing hormone (CRH)
Orexin B	Norepinephrine
Endorphins	Insulin
Ghrelin	Glucagon-like peptide
Cortisol	Cholecystokinin (CCK)
	Cocaine- and amphetamine-regulated transcript (CART)
	Peptide YY

Different sensors report the perception of satiety following food intake. First, the vagal stretch and tension sensors perceive the nutrients stored in the stomach, with the acid-sensing ion channel ASIC3 and probably additional ion channels acting as mechanosensitive transducers (Page et al., 2005). Then, neurotrophic factors are synthesised to inform about meal-taking through vagal afferent innervation of the stomach wall. Among them are the brain-derived neurotrophic factor and neurotrophin-3 that regulate long-term repercussions on energy balance (Fox, 2006). Consequently, the hormone **ghrelin** is rapidly repressed once food has been consumed (Cummings et al., 2001; Cummings, 2006), since it is the primar signal secreted by oxyntic gland cells in the mucosa of the empty stomach as a hunger signal. The presence or absence of ghrelin as a hunger or satiety signal is detected by the ghrelin receptor expressed on a

subpopulation of vagal afferent innervating neurons of the stomach in the nodose ganglia (Date et al., 2002; Burdyga et al., 2006).

Signals of ingestion are also sent from the intestine. **Cholecystokinin** (CCK) appears to be the most important hormonal signal in the upper small intestine for controlling food intake. Although peripherally derived CCK does not readily penetrate the brain (Passaro et al., 1982; Fan et al., 1997), its short-term effect on appetite has been in part attributed to stimulation of vagal sensory neurons, influencing the brainstem (Saito, Williams and Goldfine, 1981; Smith and Gibbs, 1994; Fan et al., 1997). This hormone is released from enteroendocrine cells by luminous fat and protein, but not by glucose, acting in a paracrine manner on CCK_A-receptors situated on vagal sensory nerve terminals in the mucosal lamina propria to reduce food intake (Smith, Jerome and Norgren, 1985; Raybould et al., 2006). However, CCK is also synthesized within the brain, where it is post-translationally processed into an 8 amino acid peptide (CCK-8) acting as an anorexigenic neuropeptide that reduces food intake when is centrally infused (see section 3.1) (Hirosue et al., 1993; Blevins et al., 2000).

The presence of glucose in the lumen may be communicated to the brain via the sodium-glucose cotransporter (SGLT), the release of 5-HT from another set of enteroendocrine cells, and the 5-HT3 receptor on vagal afferent nerve terminals (Morales and Wang, 2002; Wu et al., 2005; Freeman et al., 2006), or via the glucose transporter 2 (GLUT2) and T1R receptors, which are also found in the intestine (Dyer et al., 2005; Mace et al., 2007). The pancreatic β-cells also have glucose sensing capabilities. They signal glucose availability to the brain by their secretory products, insulin and amylin. **Insulin** circulates in the bloodstream in proportion to

white fat deposits serving as a sensor of body fat content (Considine et al., 1996) and acts directly on the hypothalamus and other brain areas. On the other hand, **Amylin** acts to decrease food intake and gastric emptying through receptors in the area postrema and ascending pathways to the hypothalamus and limbic structures (Lutz et al., 2001; Langhans, 2003).

With the discovery of leptin, the existence and importance of direct signals from white adipose tissue to the brain became evident (Klok, Jakobsdottir and Drent, 2007). **Leptin** levels directly correlate with body fat, and its secretions are increased after meals or periods of fasting (Friedman, 2004). This anorexigenic substance acts directly in the arcuate nucleus by inhibiting the neuropeptide Y and AgRP neurons and activating the POMC/CART neurons (see next section) to reduce food intake and increase energy expenditure signals (Abdalla, 2017).

2.1.1.2 Central regulation

The principal parts in the central regulation of the homeostatic energy balance are the hypothalamus, which integrates the afferent signals from the periphery, and the brainstem and vagus nerve that send the efferent signals to the peripheral tissue from the central nervous system (Williams and Elmquist, 2012; Kalon et al., 2016).

The hypothalamus receives sensory information from mechanical receptors in the stomach that inform about the expansion and filling of the stomach, chemical satiety signals from nutrients in the blood (such as CCK, glucose, osmolality and pH), hormonal signals from the gastrointestinal tract and adipose tissue, and central signals from the cerebral cortex (taste, smell, and vision of food) (**Figure 4**). After processing this information, efferent signals are sent from the hypothalamus to control

food intake. The hypothalamus is conformed of several interconnected nuclei that regulate appetitive control: the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the dorsomedial nucleus, the ventromedial nucleus (VMH), and the lateral hypothalamic area (LH) (Williams and Elmquist, 2012).

Specifically, the ARC and the LH are two essential areas of the hypothalamus. The **ARC** is the principal target nucleus in the hypothalamus for various satiety hormones released from the gastrointestinal tract and adipose tissue. In addition, it contains two types of neurons that project to other hypothalamic areas involved in appetite control: **orexigenic neurons** that co-express **anabolic peptides** such as neuropeptide Y (NPY) and agouti-related peptide (AgRP), and **anorexigenic neurons** that co-express the **catabolic peptides** pro-opiomelanocortin (POMC) (the precursor of melanocyte peptide-stimulating hormone, α-, β-, γ-MSH) and the cocaine- and amphetamine-regulated transcript (CART). The *orexigenic neurons* are activated by circulating factors of energetic deficit, **stimulating food intake**. Specifically, the AgRP-positive neurons target the **PVN**, the bed nucleus of the stria terminalis (BNST), the LH, and the paraventricular thalamus (PVT) to a lesser extent (Betley et al., 2013). These nuclei are inhibited by insulin, leptin and nutrients, and activated by ghrelin. Oppositely, the POMC-expressing neurons facilitate **feeding suppression**, targeting the VMH and activating the BDNF (Brain-derived neurotrophic factor) neurons (**Figure 4**) (Abdalla, 2017).

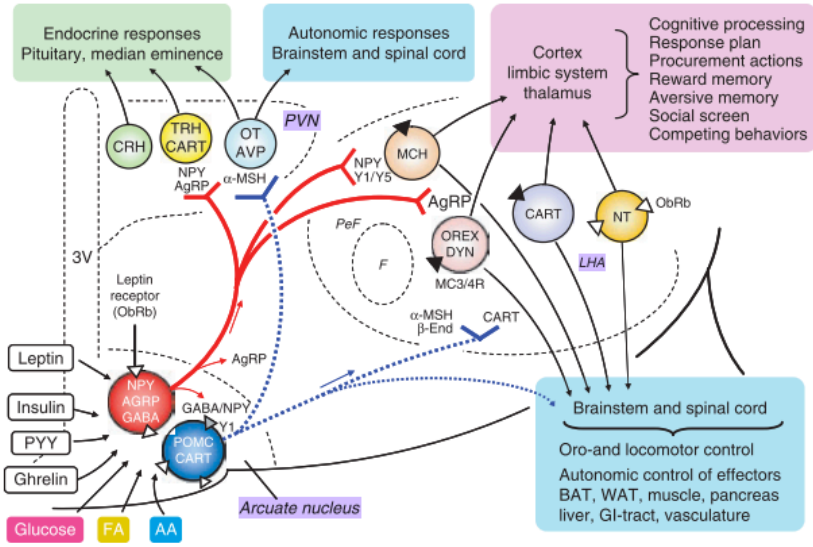


Figure 4. Hypothalamic peptidergic circuitry related to feeding and energy balance. Simplified diagram showing the two known neuron populations in the arcuate nucleus sensitive to signals of energy availability and their projections to other key neuron populations orchestrating the adaptive behavioural, autonomic, and endocrine responses. CART, cocaine- and amphetamine-regulated transcript; CRH, corticotropin-releasing hormone; GABA, γ-aminobutyric acid; MCH, melanin-concentrating hormone; α-MSH, α-melanocyte-stimulating hormone; PVN, paraventricular nucleus. Adapted from (Lenard and Berthoud, 2008).

Regarding the **LH**, this area has a role as a feeding centre by initiating the motor drives to search for food (Rossi et al., 2019), modulating the neuronal activity rapidly in an energy deficit situation (Hamburg, 1971; Burton, Rolls and Mora, 1976; Ono et al., 1981). As mentioned above, food intake regulation requires the integration of the information from both, **homeostatic** and **hedonic mechanisms**. In this sense, the LH activity modulates the hedonic quality of gustatory stimuli (Ferssiwi, Cardo and Velley, 1987) and impacts conditioned taste preference and aversion (Roth, Schwartz and Teitelbaum, 1973; Touzani and Sclafani, 2002). The LH

receives through the parabrachial nucleus (PBN) gustatory sensory information via the nucleus of the solitary tract (NTS) (Norgren, 1974; Tokita et al., 2014), and this sensitivity to food palatability, coupled with the reinforcing qualities of LH perturbations, suggests a role for the LH in promoting the consumption of palatable foods (Wiepkema, 1971). Moreover, a subset of **LH neurons** directly affects **the reward system**, projecting to the ventral tegmental area (VTA) and nucleus accumbens (NAc) (Liu et al., 2015) to modulate the activity in a conditioned feeding task and the responses to food-predictive cues consistent with reward prediction error signals (Nieh et al., 2015). The dynamics of LH neurons before and during food intake indicate that these neurons participate in both preparatory and consummatory aspects of feeding behaviour (**Figure 5**).

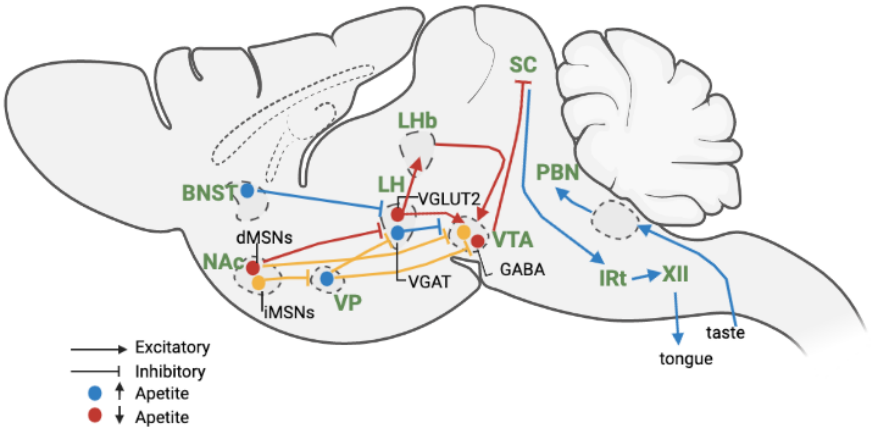


Figure 5. Summary diagram illustrating a subset of LH neuron circuits that influence food consumption. Abbreviations: BNST, bed nucleus of the stria terminalis; DA, dopaminergic; D_1R, dopamine 1 receptor; D_2R, dopamine 2 receptor; GABA, gamma-aminobutyric acid; IRt, intermediate reticular formation; LH, lateral hypothalamus; LHb, lateral habenula; NAc, nucleus accumbens; NTS, the nucleus of the solitary tract; PBN, parabrachial nucleus; SC, superior colliculus; SN, substantia nigra; VGAT, vesicular GABA transporter; VGLUT2, vesicular glutamate transporter 2; VP, ventral pallidum; VTA, ventral tegmental area; XII

hypoglossal nucleus of cranial nerve 12. Adapted from (Lenard and Berthoud, 2017).

In addition to the direct effects from the LH in the reward system, metabolic signalling from the periphery can also directly act on this circuit, modulating the dopamine (DA) reward system. The NAc and VTA express peptides and hormonal receptors for ghrelin, leptin, insulin, glucose, and glucagon-like peptide-1, among others (Domingos et al., 2011; Skibicka et al., 2011).

2.1.2 Allostatic regulation of food intake

Through the hypothalamus and caudal brainstem, the brain can integrate the information about the availability of ingested and stored nutrients and, in turn, respond generating behavioural, autonomic, and endocrine outputs. But food intake is also regulated by hedonic signals produced during ingestion. In addition, other areas of the CNS participate in the non-homeostatic or allostatic regulation of food consumption, responsible for triggering a pleasure signal together with learning and memory processes to ensure feeding. This network includes cortical areas constituting the executive system and subcortical limbic regions forming the reward system that all together exert a decisional control on food intake (Caron and Richard, 2017).

2.1.2.1 The mesocorticolimbic system in the regulation of desire and pleasure to eat

The executive brain system interacts with adjacent cortical areas and with subcortical limbic structures establishing the motivational and the pleasure value of food reward. Two main brain substrates that regulate

the reward value: the hedonic sensation or “liking” and the desire or “wanting”. The cannabinoid and opioid modulation in the brain reward system is suggested to be associated with “liking” responses, while the dopamine (DA) pathway from VTA to NAc is more associated with “wanting” or the motivation towards the pleasure of food consumption (Davis et al., 2009) (See section 2.3). This conceptual distinction emerged mainly from research on drug addiction, where stimuli that are often no longer "liked" are still intensely "wanted" (K. C. Berridge, 1996; Berridge and Robinson, 2003). Nevertheless, this conceptual and neural systems differentiation is also helpful to understand the neuropathology of some abnormal eating-related behaviours.

In the context of food intake, “liking” a food is typically followed by “wanting” it and eating it without adverse effects. However, some foods, especially high palatable foods, can exacerbate the natural rewarding effects generated during food intake, leading to abnormal consumption of energy and food even during periods of energy abundance (Kure Liu et al., 2019). Liking and wanting seem to have separate roles in promoting food overconsumption. In terms of liking, some individuals experience an exaggerated hedonic response to palatable foods (enjoy more the food) that lead to consume beyond homeostatic needs. On the other hand, processes of wanting may enhance the vulnerability to compulsive overeating through increased reactivity towards cues signalling the availability of craved food, causing self-control impairs. Thus, liking seems important in establishing the motivational properties of food, but once these are retained it is the up-regulation of wanting that promotes overconsumption in an obesogenic environment causing an “insensitivity to homoeostatic signals” and over-reactivity to external cues

associated with the craved food (Hopkins, 2016). This substantial change is used to understand how a natural reward can lead to abnormal eating-related behaviours similar to those observed for addictive substances. Notably, in the case of binge-eating disorder, one relevant factor reported in patients is the clinical and behavioural parallels with drug addicted (Davis and Carter, 2009; Avena and Gold, 2011; Davis et al., 2011; Gold, 2011). For that reason, the common neuroadaptations that drive compulsive drug- and food-seeking are investigated in this Thesis.

2.1.2.2 Similarities between the substance of abuse and binge-eating disorder

In the case of drug addiction, this mental disorder is described as a *chronically relapsing disorder* characterized by (1) **compulsion** to seek and take the drug, (2) **loss of control** in limiting intake, and (3) the emergence of a **negative emotional state** (e.g., dysphoria, anxiety, irritability) reflecting a motivational withdrawal syndrome when drug access is denied (defined as Substance Dependence by DSM) (Volkow and Li, 2005). These three stages have been represented as circular feedback that represents non-pathologic states during occasional intake (drug consumption) to malfunction results following repetitive exposition as a consequence of neuroadaptations that lead to compulsive drug-seeking (**Figure 6**).

The three hallmarks represented in the figure 6 have been widely investigated in drug addiction research, revealing three major interconnected networks: the basal ganglia (ventral tegmental area, VTA, and ventral striatum, NAc), the extended amygdala, and the medial prefrontal cortex (mPFC). The transition to addiction involves neuroplasticity in all these structures that may begin with changes in the

mesolimbic dopamine system and a cascade of neuroadaptations from the ventral striatum to dorsal striatum and orbitofrontal cortex and eventually dysregulation of the prefrontal cortex, cingulate gyrus (related to conditioning/habits, motivation, and executive processes), and extended amygdala (Koob and Volkow, 2009). Nevertheless, similar behavioural malfunctions are described for binge-eating patients: 1) eat significantly more calories than obese non-binge eaters (**compulsive overeating**) (Galanti, Gluck and Geliebter, 2007); (2) experiencing a **loss of control over eating** during the episodes; and (3) **feeling disgusted** with oneself and depressed after episodes (American Psychiatric Association, 2013). In addition, *high relapse rates* during weight-loss control programs are especially reported in obese that also suffer binge-eating disorder, indicating "withdrawal" symptoms in periods of food restriction that lead to relapse (Wilson, Nonas and Rosenblum, 1993). Thus, the binge consumption can activate specific neuronal pathways similar to that after drug consumption leading to corresponding signs of dependence (Avena et al., 2015). The important overlapping between compulsive drug-seeking reported for drug addiction and compulsive food-seeking described in binge-eating disorder is further evidence of the potent reinforcing power of palatable food that can over-activate neurobiological substrates similarly that drugs do it (Schultz, 1998; Stuber, Wightman and Carelli, 2005).

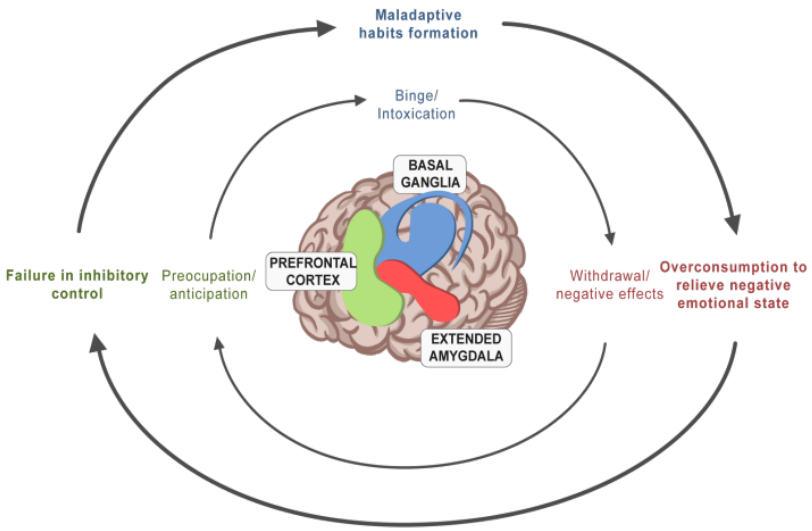


Figure 6. Neurocircuitry schematic illustrating the brain region according to the three stages of the addiction cycle that promote drug and food-seeking behaviour. The Inner ring represents the occasional exposition to substances. The external ring represents malfunction results after over-exposition

2.2 The brain's reward system

The rewarding effects produced by both natural reinforcers (sex, water and food) and substances of abuse are a consequence of their action in the brain's reward system. Within this system, two principal pathways that compromise the projecting dopaminergic neurons from the VTA to limbic (**mesolimbic pathway**) and cortical areas (**mesocortical pathways**) (Chi Yiu Yim and Mogenson, 1980; D'Ardenne et al., 2008; Phillips, Vacca and Ahn, 2008). However, these two principal pathways are strongly overlapped and they are often collectively referred to as the **mesocorticolimbic system** (Wise, 2004).

The perception and appraisal of rewarding or aversive stimuli in the environment are mainly regulated by the fluctuating DA levels in the

DAergic projecting neurons from the VTA. For example, the exposition to a **positive reinforcement** activates the release of DA from the VTA to NAc to promote the feeling of pleasure, along with areas related to emotional states and the formation of associative reward-related memories (such as amygdala and hippocampus). The feeling of pleasure, in turn promotes goal-direct behaviour to obtain the reward caused by appetitive stimuli (Koob, 2015; Koob and Volkow, 2016) regulated by frontal cortical regions that mediate the control of executive functions. On the other hand, **aversive stimuli** can also modulate the same circuit decreasing the amount of DA at the synaptic levels, contributing to negative reinforcement induced by aversive emotional states (Koob, 2017). Thus, fluctuating DA levels from the VTA contribute critically to coding the motivational value or silencing of a given stimulus (Bromberg-Martin, Matsumoto and Hikosaka, 2010), together with the complex interconnected network that involves glutamatergic, GABAergic, cholinergic and dopaminergic transmission from the related areas (**Figure 7**).

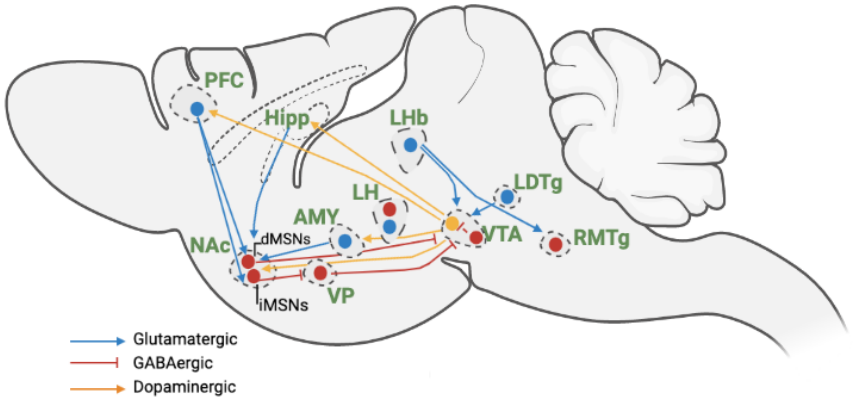


Figure 7. The mesocorticolimbic circuitry. A simplified schematic diagram of the mesocorticolimbic circuitry in rodent brain highlighting the major dopaminergic,

glutamatergic and GABAergic connection to and from the ventral tegmental area (VTA) and nucleus accumbens (NAc). Amy, amygdala; Hipp, hippocampus; LH, lateral hypothalamus; mPFC, medial prefrontal cortex; LHb, lateral habenula; LDTg, lateral dorsal tegmentum; RMTg, rostromedial tegmentum. Adapted from (Russo and Nestler, 2013).

2.2.1 Ventral tegmental area

The VTA is a heterogeneous brain area composed of dopaminergic (~60%) and GABAergic (~30-35%) projection neurons, whose activity is regulated by inputs from various brain regions and by local GABAergic (~30-35%) and GLUTergic (2-3%) interneurons (Margolis et al., 2012; Morales and Margolis, 2017). Although most VTA neurons exclusively release dopamine, GABA or glutamate, specific VTA neurons can co-release several neurotransmitters with different combinations: dopamine and glutamate (TH-VGLUT2), dopamine and GABA (TH-VGAT), or glutamate and GABA (VGLUT-VGAT), as well as in combination with other neurotransmitters and peptides such as CCK (Seroogy et al., 1988; Jayaraman et al., 1990) and BDNF (Seroogy et al., 1994) among others. To better understand how these different VTA neurons integrate information to produce reward and aversion behaviours independently, it is necessary to determine the organization of connectivity in the VTA and the functional nature of the synapses established by these neurons in downstream brain structures. However, the cytoarchitecture of the VTA is widely sophisticated, and the total complexity circuitry that integrates the VTA is still unknown (Morales and Margolis, 2017). For that reason, the VTA circuits involved in reward- and aversion-related behaviours will be summarised.

Inputs onto VTA neurons

Given the apparent participation of VTA dopamine neurons in different aspects of behaviour, the activity of this specific subpopulation of neurons is especially relevant. Accumulating evidence shows that putative VTA dopamine neurons (identified by their electrophysiological properties) are activated in response to unpredicted rewards, shift their activation to cues that predict reward following learning and transiently decrease their firing when an expected reward is omitted (Schultz, Dayan and Montague, 1997; Bromberg-Martin, Matsumoto and Hikosaka, 2010; Cohen et al., 2012). The activity of the VTA dopamine neurons is regulated by several brain regions that directly innervate the VTA, including glutamatergic projections from the laterodorsal tegmental nucleus (LDT), the lateral habenula (LHb), and the prefrontal cortex, and inhibitory inputs of GABAergic projections from the rostromedial tegmental nucleus (**Figure 8**) (Cooper, Robison and Mazei-Robison, 2017).

Previous studies using electrical stimulation demonstrated that the laterodorsal tegmental nucleus promotes burst firing of putative VTA DA neurons and increases DA release in the NAc (Forster and Blaha, 2000; Lodge and Grace, 2006). Consistent with the idea that DA release in the NAc promotes reward (Witten et al., 2011; Steinberg and Janak, 2013), Lammel et al. (2012) demonstrated that phasic light activation of the laterodorsal tegmental nucleus inputs within the VTA elicits conditioned place preference (CPP), which was prevented by infusion of DA receptor antagonists into the NAc.

Similar approaches were used to study the connectivity and behavioural functions of the inputs to the VTA from the lateral habenula. It is thought that this area is critical for mediating behavioural responses

to aversive stimuli and when expected rewards do not occur (Hikosaka, 2010). This dual effect was demonstrated by optogenetic activation of lateral habenula axons in acute slices that generate excitatory synaptic currents in GABAergic neurons in the rostromedial tegmental nucleus (RMTg) (Lammel et al., 2012; Stamatakis and Stuber, 2012), also called as "tail" of the VTA (Kaufling et al., 2009). Furthermore, these same inputs from the lateral habenula to the RMTg GABAergic neurons generate inhibitory synaptic currents in DA cells projecting to NAcShell, which is thought to influence the reward-related responses. These results reported an indirect effect of the lateral habenula projecting neurons onto the VTA DA neurons regulation. However, direct inputs from the lateral habenula also made excitatory synaptic connections onto the VTA DA neurons that project to the mPFC, inducing aversive behaviour, which was prevented by infusion of a DA receptor antagonist into the mPFC (Lammel et al., 2012). These findings suggest that diverse afferent inputs to the VTA affect different DA and non-DA neuron subpopulations and VTA "microcircuits", influencing the behaviour differently.

Outputs from VTA neurons

In a general view, the VTA has two main DA output routes, one to the NAc (**mesolimbic**) and the other to the PFC (**mesocortical**), to coordinate reward-seeking behaviour (**Figure 8**). Reinforcement phenomena are caused by stimulation of the mesolimbic axis that increases the extracellular DA content in the NAc, which is directly related to the intensity of the "pleasure" sensation (Volkow, Wang and Baler, 2011; Oleson and Cheer, 2012). However, other non-dopamine VTA outputs have been proposed to contribute to reward- or aversion-related

behaviour (Stamatakis et al., 2013; Qi et al., 2016). For example, selective optogenetic activation of VTA glutamate neurons that project to NAc in mice produces aversion through synaptic activation of parvalbumin-expressing GABA interneurons in the NAc, which drives GABA release onto medium spiny neurons (Qi et al., 2016). By contrast, VTA dopamine neurons reduce GABA release in the NAc, increasing the motivational drive (measured as an increase in optical self-stimulation inside the NAc) (Berrios et al., 2016).

Activating the mesocortical pathway is critical for cognitive control, motivation, and emotional response regulation (Volkow, Wise and Baler, 2017). This role in cognitive control was demonstrated by the developmental elimination of VTA dopamine neurons that project to the mPFC in mice, resulting in a loss of cortical inhibition mediated by mesocortical dopamine- and glutamate-releasing inputs neurons (Kabanova et al., 2015). However, VTA dopaminergic neurons also innervate other regions, including the amygdala and hippocampus, where aversive states and contextual associations of drug-related cues are processed, respectively (Koob and Volkow, 2009).

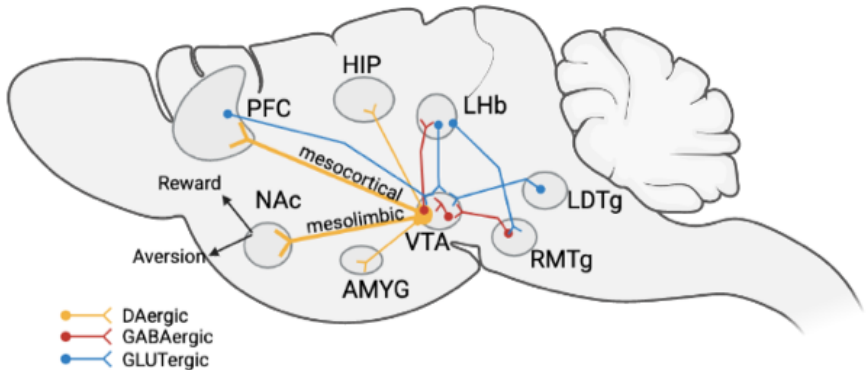


Figure 8. Schematic representation of the complex VTA microcircuitry showing the main outputs and inputs. PFC, prefrontal cortex; HIP, hippocampus; LHb,

lateral habenula; LDTg, laterodorsal tegmental nucleus; RMTg, rostromedial tegmental nucleus; VTA, ventral tegmental area; AMYG, amygdala; NAc, nucleus accumbens. Adapted from Cooper et al., 2017.

2.2.2 Nucleus accumbens

The striatum is a central connectivity hub located in the forebrain, processing motivation, affect, cognition, and sensorimotor information arising from different systems to produce a behavioural action output. Even though the entire striatum appears to follow the same fundamental information influx-outflux pattern, many lines of evidence suggest that the striatum can be divided into discrete subregions based on morphological and functional differences: **dorsal** and **ventral striatum** (Castro and Bruchas, 2019). These striatal subregions share many anatomical and functional similarities, but they are highly heterogeneous regions and can be differentiated a relative distribution of different cell types (matrix and patch) (**Figure 9**) (Gerfen, Herkenham and Thibault, 1987; Gerfen, 1992; Crittenden and Graybiel, 2011; Watabe-Uchida et al., 2012; Brimblecombe and Cragg, 2017).

Specifically, the ventral striatum (so-called **nucleus accumbens**) is an important hub of communications-processing and the initial target for natural rewards and drugs reinforcing effects by stimulating dopamine transmission (di Chiara and Imperato, 1988; Nestler, 2005; Schultz, 2006; Volkow et al., 2011; Volkow, Wang and Baler, 2011; du Hoffmann and Nicola, 2014). This key node has critical functional connections between limbic system structures, such as the amygdala and hippocampus, and motor system, including the basal ganglia (Mogenson, Jones and Yim, 1980). The NAc is composed of three major subnuclei: the core (NAcCore), the lateral shell, and the medial shell (NAcShell) (**Figure 9**) (Záborszky et

al., 1985). This complex brain area is composed of five main neuronal populations: three different interneurons and two projecting ones. The interneurons constitute around 5% of the total neuronal population in the striatum (Kawaguchi, 1993; Tepper et al., 2010), and have been classified as (1) fast-spiking (FSI) and parvalbumin-releasing (PV), (2) persistent low threshold and somatostatin-releasing (SOM), and (3) tonically active and acetylcholine-releasing (CIN) interneurons, based on their firing patterns and neurotransmitter content. The projection population or medium spiny neurons (MSNs or SPNs) (Ramon y Cajal, 1911; Wilson et al., 1983; Meredith et al., 2008) are GABAergic neurons that account for ~95% of all neurons in the NAc. Output projecting areas, neurochemical content and receptor markers divide these two neuronal subpopulations into the "direct pathway" (dMSNs) that expresses dopamine receptor type-1 (D_1-MSNs), dynorphin and substance P, and the "indirect pathway" (iMSNs) that expresses dopamine receptor type-2 (D_2-MSNs), adenosine 2A receptors (A2A), and enkephalin (**Figure 9**) (Castro and Bruchas, 2019).

The NAc subnuclei have different anatomical connectivity (different out-input pathways) (**Figure 10**) (di Chiara and Imperato, 1988; Ikemoto, 2007; Scofield et al., 2016), which also generate distinct behavioural functions. The core is assumed to integrate learning reward-cue associations, responding to motivating cues, making impulsive decisions, and initiating motor actions. In contrast, the shell (lateral and medial) is involved in reward prediction, affective processing, and drug relapse (Volkow, Wise and Baler, 2017).

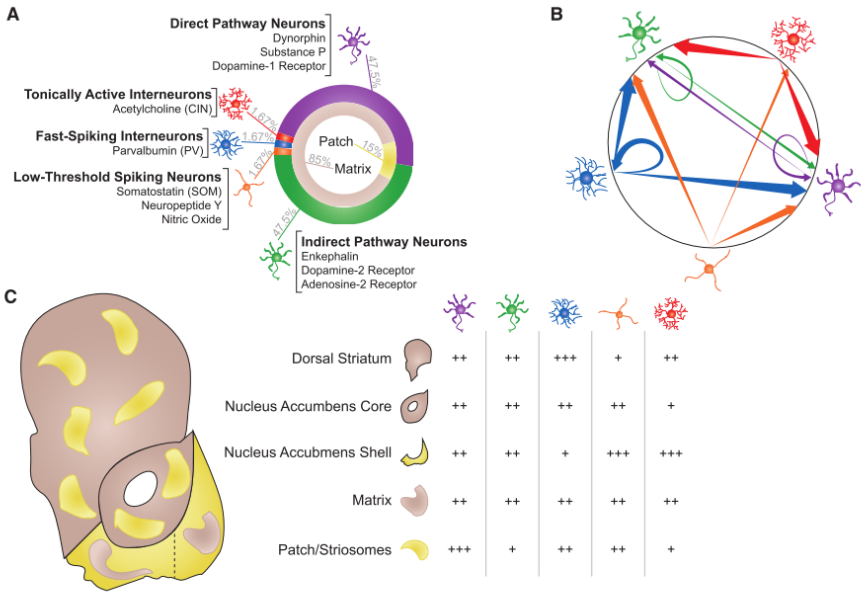


Figure 9. Basic Structural Anatomy of the Striatum. A) Ring charts showing relative expression (grey text) of cell types (outer ring) or patch-matrix (inner ring). Cell types are labelled with known and exclusive markers for each population. **B**) Intra-striatal connectivity schematic showing preferential connections between cell types. Arrows signify projection targets, circled arrows signify synapses onto other neurons of the same type. Cell populations: purple, direct pathway; green, indirect pathway; blue, fast-spiking interneuron; orange, low-threshold spiking interneurons; red, tonically active interneurons. Arrow thickness: thin, low connectivity; medium, moderate connectivity; thick, strong connectivity. **C**) Bottom left: schematic of the rodent striatum, dividing dorsal striatum, core, and shell into separable subregions (lateral and medial, divided by a dashed line). Matrix (brown) and patch (yellow) are shaped and distributed to show relative expression in each subregion. Right: relative distribution of different cell types within each region of the striatum. Direct pathway neurons, purple; indirect pathway neurons, green; tonically active interneurons, red; fast-spiking interneurons, blue; low-threshold spiking interneurons, orange. +, low relative expression; ++, average relative expression; +++ high relative expression. Adapted from (Castro and Bruchas, 2019).

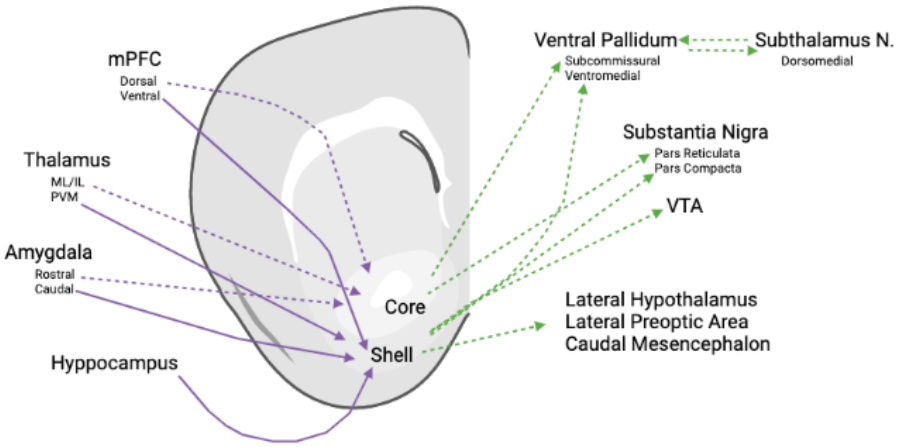


Figure 10. Schematic diagram of the inputs and outputs of the shell and core of the nucleus accumbens. Dopaminergic, serotonergic, and noradrenergic inputs have been omitted from the drawing. ML/IL, midline and intralaminar thalamic nuclei; mPFC, medial prefrontal cortex; PV, paraventricular thalamic nucleus; VTA, ventral tegmental area (Basar et al., 2010).

2.2.2.1 "Direct" and "indirect" pathways

The "direct" and "indirect" pathways labels describe the downstream efferent destinations. However, recent studies suggest that the classical dichotomy between the roles of D_1-MSNs and D_2-MSNs as synonymous with direct and indirect pathways in the dorsal striatum is not conserved in the NAc. Traditionally, in the dorsal striatum, the D_1-MSNs from the direct pathway that extends axonal projections to the internal part of the globus pallidus (GPi) and substantia nigra pars reticulata (SNpr). These GABAergic projections inhibit the GABAergic neurons in the SNpr, causing disinhibition of the thalamic glutamatergic neurons. On the other hand, the D_2-MSNs form the indirect pathway because they innervate the SNpr indirectly via the external part of the globus pallidus (GPe) and the subthalamic nucleus (STN). The disinhibition of the STN glutamatergic

projection to the SNpr/GPi causes an inhibition of the thalamus caused by GABAergic projection from the SNpr/GPi (**Figure 11**). The resulting movements are governed by the proper balance of both pathways (Kreitzer and Malenka, 2008; Gerfen and Surmeier, 2011).

Given the similarities in anatomical features and cellular distributions, direct and indirect outflows in the NAc were initially described as in the dorsal striatum but targeting different areas) associated with reward-related behaviours (**Figure 11)**. The direct pathway (D_1-MSNs) innervates the midbrain in the substantia nigra (SN) and ventral tegmental area. Meanwhile, the indirect pathway targets pallidal and hypothalamic structures (Nicola, 2007; Kupchik and Kalivas, 2017a), and then, these innervations target the midbrain. However, the MSN outputs in the NAc follow a direct and indirect division but may not be divisible by D_1-D_2 expression patterns (**Figure 11**). Optogenetic and tracing tools studies revealed that a significant proportion of D_1-MSNs also innervates the ventral pallidum, while some D_2-MSNs project directly to the thalamus. These recent results compromise the classical direct and indirect pathway differentiation and highlight the ventral pallidum as an important hotspot of reward circuitry given that D_1-MSNs may participate in an “indirect pathway”-like manner innervating the ventral pallidum, and the D_2-MSNs may be involved in the “direct pathway” regulation (**Figure 11**) (Kupchik et al., 2015; Kupchik and Kalivas, 2017b; Klawonn and Malenka, 2018).

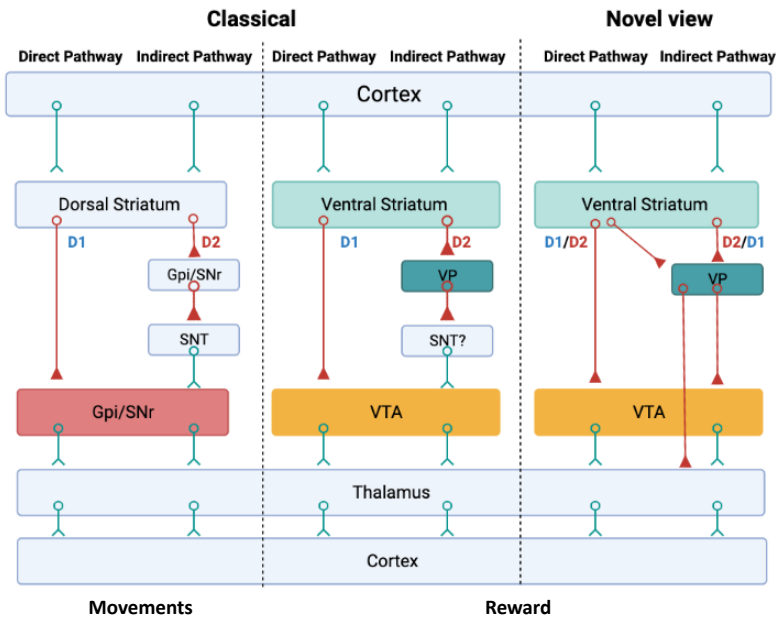


Figure 11. Schematic representation of the canonical view for the direct and indirect pathway in the dorsal striatum (left). A traditional and novel view of the direct and indirect pathway in the ventral striatum (middle and left). GPe and GPi, external and internal globus pallidus; VP, ventral pallidum; SNpr, substantia nigra pars reticulata; STN, subthalamic nucleus; D_1 and D_2 dopaminergic receptors. Excitatory projections in green and inhibitory projections in red. Adapted from (Gerfen and Surmeier, 2011; Mannella et al., 2013)

2.2.2.2 Indirect pathway in the nucleus accumbens: role of ventral pallidum in reward-related behaviour and food intake

The NAc innervates different brain areas, such as the substantia nigra (SN), VTA, hypothalamus, VP and brainstem. However, recent studies have paid particular attention to overeating highly rewarding foods of two important interconnected structures that modulate reward processing, the NAc and the VP (Gendelis, Inbar and Kupchik, 2021). Understanding how dysfunctions in brain regions involved in reward-seeking and consumption

may drive overeating-related behaviours and obesity and the similarities between them and addiction is only now starting to be revealed.

In this sense, recent studies with cocaine-related behaviour reported that the cocaine-induced changes in synaptic plasticity could be cell type-specific, as are their outputs, which likely contribute to their differential effects on cocaine-related behaviour (Smith et al., 2013). This fact has recently been shown to be the case in the VP, a robust target of both D_1 and D_2 NAc MSNs in the indirect pathway, where cocaine exposure simultaneously potentiated D_1 NAc-VP output but depressed D_2 NAc-VP output (Creed et al., 2016). Furthermore, these changes in plasticity influenced different elements of addictive-like behaviours. Restoration of function at D_1 NAc-VP synapses removed cocaine locomotor sensitization, whereas function recovery at D_2 NAc-VP synapses corrected cocaine withdrawal-induced anhedonia, implying that NAc D_1 and D_2 MSN afferents to VP regulate different behavioural features associated with cocaine addiction (Creed et al., 2016). This study emphasizes not only the differences between D_1 and D_2 MSN circuits but also the significance of the NAc-VP circuit in cocaine-related behaviours, a topic of great recent interest (Stefanik et al., 2013; L. Wang et al., 2014; Root et al., 2015; James and Aston-Jones, 2016; Heinsbroek et al., 2017).

The VP contribution to several motivation behaviours depends upon the participation of GABAergic neurons belonging to individual VP subregions (ventral pallidum ventromedial, VPvm, and ventral pallidum dorsolateral, VPdl) and non-GABAergic neurons, which affect discrete neuronal circuits. Thus, the NAcShell innervation to the GABAergic VPvm neurons is involved in discriminating the stimulus conditions of reward/drug acquisition, consumption, and working memory. In contrast,

the VPdl neurons innervated by NAcCore projection mediate motivated reward behaviour, such as drug-seeking responses (Root et al., 2015). Repeated exposure to a reward (such as drug use) impairs the standard integrative capabilities of this brain area, resulting in motivation and reward output deficits. In addition, the VP is also involved directly in consummatory behaviours and food intake. Activation of $GABA_A$ receptors in VP decreases food intake by intra-VP injection of the muscimol (Shimura, Imaoka and Yamamoto, 2006; Taha et al., 2009) and conversely, intra-VP injection of the $GABA_A$ receptor antagonist bicuculline increases food intake, particularly food with a high-fat content (Stratford and Kelley, 1999; Smith and Berridge, 2005; Inui, Shimura and Yamamoto, 2007; Covelo et al., 2014). In addition to these functions, the VP neurons seem to be also sensitive to cues related to hedonic taste reactions (Tindell et al., 2009) and cue-induced feeding (Leung and Balleine, 2013). Therefore, this particular pathway has become an interesting target for investigating motivational features for highly caloric and palatable food-seeking.

2.2.3 Prefrontal cortex

The PFC is a brain area involved in several executive functions, including cognitive, emotional and motivational processes. These functions are associated with control behaviour, such as response inhibition, planning, attention, and decision-making (Miller and Cohen, 2001). Dysregulation of these functions leads to a loss of self-control, driving compulsive behaviour development (a hallmark of addiction and some eating disorders) (Goldstein and Volkow, 2011).

Two subregions can be differentiated in the PFC: the medial prefrontal cortex (mPFC) and the orbitofrontal cortex (OFC) (Koob and

Volkow, 2009). Regard mPFC, the cytoarchitecture of this area is composed of 80% excitatory glutamatergic pyramidal projection neurons and 20% inhibitory GABAergic interneurons (Pistillo et al., 2015). Like other cortical areas, the mPFC possesses a laminar (L) architecture that forms intracortical circuits between excitatory pyramidal neurons in the superficial layers (L2/3) and other pyramidal and GABAergic neurons. Such excitatory projections are also sent from deeper layers (L5/6) to subcortical locations, including the VTA and NAc. Due to this laminar structure, each layer can receive afferents from different areas. Deep pyramidal neurons in L5 and 6 receive afferent projections from VTA dopaminergic neurons, while superficial pyramidal neurons in L2, 3, and 5 receive inputs from other cortical areas, thalamus, basolateral amygdala (BLA), and hippocampus. Thus, the VTA-PFC loop (mesocortical circuit) evaluates the salience motivational significances associated with context and stimuli (such as drug- and food-associated context) (**Figure 12**) (Douglas and Martin, 2004). In addition, the mPFC is divided into several sub-regions that seem to play distinct functional roles: the medial precentral, anterior cingulate, prelimbic (PL), and infralimbic (IL) cortex (Heidbreder and Groenewegen, 2003). These three sub-regions of the mPFC have been widely related to drug addictive processes. For instance, the anterior cingulate cortex is primarily engaged in attentional selectivity and discriminating learning and the creation of impulsive actions (Perry et al., 2011) and cocaine reinstatement caused by signals (Kalivas and Volkow, 2005). In the case of PL and IL, initial studies in cocaine addiction reported that both areas had a differentiated and opposite function in cocaine-seeking behaviour. Whereas PL promotes cocaine-seeking, IL suppresses such behaviour after extinction (Moorman et al., 2015).

However, depending on the behavioural environment, the type of substance, and previous history of drug consumption, both PL and IL cortices drive and inhibit drug-seeking, implying that various subcircuits within each of these mPFC areas may have distinct behavioural functions (Riga et al., 2014; Moorman et al., 2015).

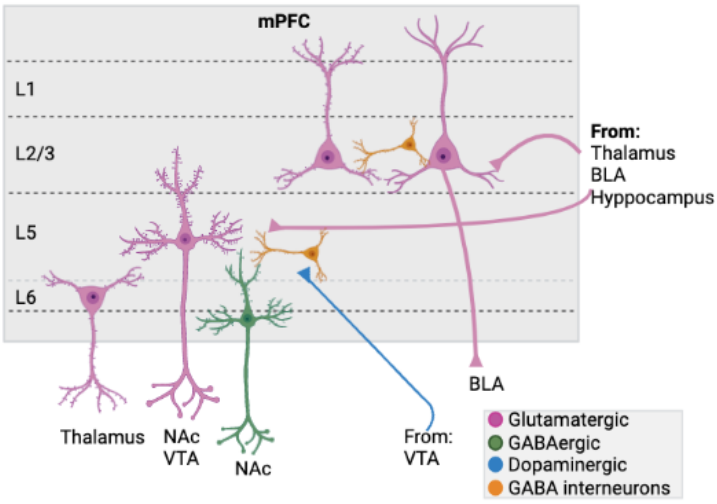


Figure 12. Simplified diagram of the connectivity in the rodent mPFC. The pyramidal neurons in superficial layers (L2/3) are mainly cortico-cortical neurons. The pyramidal neurons in deep layers (L5 /6) are cortico-subcortical neurons mainly projecting to VTA and NAc. Pyramidal neurons in L5 and 6 receive projections from VTA DA neurons while pyramidal neurons in L2, 3 and 5 receive functional inputs from other cortical areas, thalamus, BLA and hippocampus. NAc, Nucleus accumbens; VTA, ventral tegmental area; BLA, basolateral amygdala; FS, fast-spiking, NFS, non-fast spiking. Adapted from (Pistillo et al., 2015).

On the other hand, the OFC role is still unclear. Some authors argued that OFC is involved in appropriate flexible behaviour and goal-directed decision making (Wikenheiser and Schoenbaum, 2016) and is also considered a crucial area in the inhibitory control of inappropriate responses in a current context (Perry et al., 2011). However, more recent studies argued against response inhibition as the primary role of the OFC,

suggesting a more relevant role in associative learning and decision-making (Stalnaker, Cooch and Schoenbaum, 2015).

Altogether, the frontal areas control the basal ganglia and extended amygdala microcircuits by sending glutamatergic projections top-down to numerous brain areas such as the VTA, NAc, dorsal striatum, hippocampus, and amygdala (Parsons and Hurd, 2015). Specific "Go circuits" and "Stop circuits" have been identified based on whether their recruitment facilitates or hinders the transition to addictive behaviours (Bock et al., 2013; Picciotto, 2013). The OFC neurons that link directly to the medial striatum cluster, which comprises the NAcCore and the medial wall of the dorsal striatum, are activated by the "Go circuit" (Pascoli et al., 2018; Hu et al., 2019; Wall et al., 2019). On the other hand, the "Stop circuit", in turn, stimulates excitatory synaptic transmission in PL mPFC neurons projecting to the NAcCore, which inhibits compulsive-like behaviour (**Figure 13**) (Hu et al., 2019; Domingo-Rodriguez et al., 2020). An imbalance between both circuits is crucial in the transition to addiction and compulsive-like behaviours.

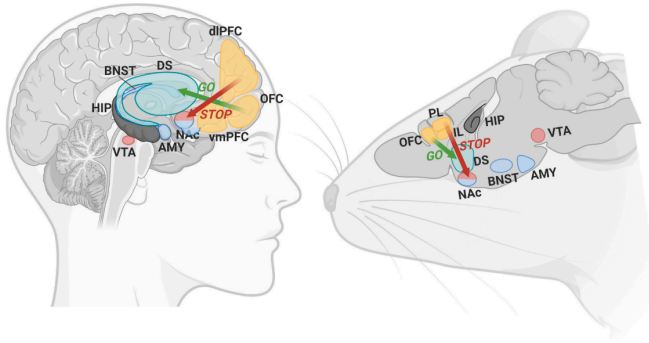


Figure 13. Schematic representation of mouse brain areas involved in reward sensitivity, conditioning, emotional processing, and inhibitory control circuits. The loss of control or compulsivity seems to result from an imbalance between the "Stop" and "Go" top-down cortical circuits with a hypofunction of the PL-ventral striatum network and hyperactivity of the OFC-dorsal striatum networks.

VTA, ventral tegmental area; NAc, nucleus accumbens; DS, dorsal striatum; AMY, amygdala; BNST; bed nucleus of the stria terminalis; HIP, hippocampus; dlPFC, dorsolateral prefrontal cortex; vmPFC, ventromedial prefrontal cortex; OFC, orbitofrontal cortex; PL, prelimbic; IL, infralimbic. Adapted from (Maldonado et al., 2021).

2.3 Modulation of the reward system

Three main endogenous signalling systems modulate the brain's reward circuitry: opioid, dopaminergic (DA) and endocannabinoid (EC) systems. They all interact to integrate the hedonic values of naturally pleasurable stimuli.

The rewarding properties of a natural compound, such as food, can be divided into two different **functional components:** "liking" (pleasure/palatability, the hedonic component) and "wanting" (appetite/incentive motivation), and they can be manipulated and measured separately. Specific neural substrates process these two separated functional components. Mediation of liking related to food reward involves neurotransmitter systems such as opioid and GABA/benzodiazepine systems and anatomical structures such as the ventral pallidum and the brainstem primary gustatory relays. On the other hand, mediation of wanting related to food reward involves mesotelencephalic dopamine systems, nucleus accumbens division and amygdala (K. Berridge, 1996).

2.3.1 Opioid signaling: the brain substrate of “liking”

The endogenous opioid system is intimately involved in hedonic functions and incentive motivation, as well as in generating pleasurable sensations when consuming palatable foods (Berridge et al., 2010; Nummenmaa and Tuominen, 2018). It is composed of endogenous opioids (endorphins,

enkephalins, and dynorphins) that activate opioid receptors (mu, delta, and kappa receptors). In addition, the opioid system is implicated in assigning hedonic values to rewards and integrating information related to rewards to guide decision-making and execution of goal-directed behaviour (Volkow, Michaelides and Baler, 2019).

The activation of the μ-opioids receptor (MOR) by morphine injection into the mesolimbic reward system or by receptor stimulation in the NAcShell increases pleasure (Bozarth and Wise, 1981) obtained from foods and may also promote feeding (Peciña and Berridge, 2005). Similarly, opioid agonists and antagonists increase and decrease food intake and hedonic reactions to appetizing meals, respectively (Glass, Billington and Levine, 1999; Yeomans and Gray, 2002; Giuliano et al., 2012; Ziauddeen et al., 2013), whereas inverse MOR agonists reduce the hedonic impact of feeding (Nathan et al., 2012). These findings are supported by human imaging studies that reveal that eating causes an endogenous opioid release in the brain's reward circuit (Burghardt et al., 2015; Tuulari et al., 2017), possibly contributing to the pathophysiology of obesity. In fact, it has been hypothesized that repeated overstimulation of the MOR after prolonged consumption of high palatable food may result in a long-term downregulation of MOR and the subsequent requirement of larger amounts of food to produce the same hedonic effect. This mechanism is suggested as a neurobiological process that supports overeating and compulsive-like eating with significant differences between obese and obese with binge eating disorder (Davis et al., 2009).

2.3.2 Dopamine signalling: the brain substrate of “wanting”

Dopamine (DA) is a catecholamine neurotransmitter important for many physiological functions, including motor control, mood, and reward processing. Given the breadth of DA's vital tasks, it is not surprising that dopaminergic malfunction has been related to a variety of human illnesses, including Parkinson's disease, Tourette's syndrome, schizophrenia, obsessive-compulsive disorder, and addiction (Tritsch and Sabatini, 2012), as well as in the regulation of feeding of palatable foods (Volkow, Wang and Baler, 2011). We will focus on the role of DA in the reward system modulating motivational aspects associated with eating behaviours. In this sense, the involvement of DA in reward is more complex than the mere encoding of hedonic value. For example, the "unexpected" reward can cause a different sensation of pleasure if it is greater or smaller than expected. This fact highlights the importance of DA signalling in reward-related processing in modulating reinforcement-seeking motivation rather than the sensation of pleasure itself.

Most dopamine neurons in the midbrain of humans, monkeys, and rodents signal a **reward prediction error**, which means that the DAergic neurons' activity varies according to the reward obtained compared to predicted expectancies based on previous experiences (Schultz, 2016). In this sense, the DAneurons are more active with a greater reward than predicted reward (**positive prediction error**) and remain at baseline for fully predicted reward. Oppositely, they show depressed activity with a smaller reward than predicted (**negative prediction error**), motivating behavioural changes to obtain the predicted reward again. This process is described for both drugs and high palatable food that modulated the DA signals in the brain's reward system in response to reward cues (Schultz,

2010). In the case of palatable food, upon the first exposure to a food reward (or an unexpected reward), the firing of DAneurons in the VTA increases with a resulting increase in DA release in NAc (Norgren, Hajnal and Mungarndee, 2006) (positive predictor error). With **repeated exposure to the food reward**, the DA response habituates and is gradually transferred onto the stimuli associated with the food reward (such as the smell of food), which is then processed as a predictor of reward (becoming a cue that is conditioned to the food reward) (Epstein et al., 2009; Schultz, 2010).

2.3.2.1 Dopaminergic neurons

DA is expressed both in the mammalian central nervous system and in various peripheral tissues. Within the brain, four major dopaminergic networks have been identified: (**I**) the **nigrostriatal pathway**, which includes neurons projecting from the substantia nigra pars compacta to the caudate-putamen in the dorsal striatum, whose degeneration causes Parkinson's disease (Lang and Lozano, 1998a, 1998b); (**II**) the **mesolimbic pathway**, which includes projections from the ventral tegmental area to the nucleus and olfactory tubercles, mainly involved in reward processes (Koob, Sanna and Bloom, 1998); (**III**) the **mesocortical pathway**, that originates from neurons in the tegmentum and projects to the frontal cortex, mainly in the medial prefrontal regions, implicated in cognitive functions and motivation (le Moal and Simon, 1991); (**IV**) the **tuberoinfundibular pathway** that arises in the arcuate nucleus and ends in the median eminence of the hypothalamus, implicated in the neuroendocrine regulation and the prolactin release (**Figure 14**) (Vallone,

Picetti and Borrelli, 2000; Maharajan et al., 2001; Tarazi, 2001; Beaulieu and Gainetdinov, 2011).

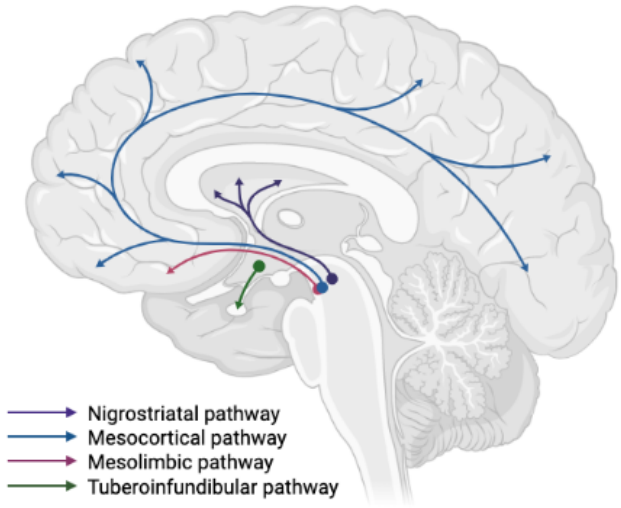


Figure 14. Dopaminergic pathways in the brain. Four dopaminergic pathways are presented: the nigrostriatal pathway, mesolimbic pathway, mesocortical pathway, and tuberoinfundibular pathway. VTA, ventral tegmental area; SN, substantia nigra; NAc, nucleus accumbens.

When DA neurons fire, they can do so in a stable irregular tonic mode (low frequency, 1-8 Hz) or in a transitory (500 msec) phasic mode (high frequency, >15 Hz) with a burst of action potential. The low-frequency spontaneous firing suggests that each neuron supplies a basal DA tone to many target neurons, crucial for quick sensitivity to external stimuli. Exposure to salient (reinforcing, novel, unexpected, or aversive) stimuli causes changes in firing from low frequencies to burst phasic firing at high frequencies, implicated in reinforcement learning (Volkow, Wise and Baler, 2017). Intrinsic membrane characteristics of DA neurons produce tonic firing, which results in DA release (in the range of nM) from extrasynaptic release sites. The activation of the high-affinity DA receptors

(DA-autoreceptors), which determine motivational arousal, is induced by these modest DA levels (Ford, 2014). Phasic spiking, on the other hand, is dependent on glutamate receptor activation and voltage-gated ionic channels and results in a sizeable extracellular DA release (in the range of μM) in the synaptic cleft, which stimulates the low-affinity postsynaptic DA receptors, resulting in response to behaviorally salient stimuli (Dreyer et al., 2010). After that, DA is quickly eliminated from the synapse by the DA transporter (DAT), and the signal is ended.

2.3.2.2 Dopamine receptors: signalling and distribution

DA acts by binding to five distinct receptors members of the G-protein coupled receptor superfamily. These receptors are formed by seven hydrophobic transmembrane sections and a vital third intracytoplasmic loop that interacts with several types of G-proteins and diverse effector molecules (Missale et al., 1998; Tarazi, 2001; Beaulieu and Gainetdinov, 2011). According to the biochemical and pharmacological features, these receptors are divided into the D_1-like family, including D_1 and D_5 receptor subtypes (D_1R and D_5R), and the D_2-like family, comprising D_2, D_3 and D_4 receptor subtypes (D_2R, D_3R and D_4R) (Jaber et al., 1996). Individual members of the same subfamily have similar structural properties, but sequence variants determine ligand affinity and signal transduction pathway coupling differences. D_2-like receptors have a 10- to 100-fold higher affinity for DA than D_1-like receptors, with D_3 and D_4 receptors having the highest sensitivity for DA and D_1 receptors with the lowest. However, D_1 and D_2 receptors can exist in both high and low-affinity states, and their high-affinity states have similar nanomolar affinities for DA (**Table 2**) (Beaulieu and Gainetdinov, 2011).

Table 2. Basic characteristics of DA receptors. (Modified from Tritsch and Sabatini, 2012)

	D_1-LIKE FAMILY		D_2-LIKE FAMILY		
DA receptor subtype	D_1R	D_5R	D_2R	D_3R	D_4R
Gene name	*Drd1*	*Drd5*	*Drd2*	*Drd3*	*Drd4*
Number of introns	0	0	6	5	3
Splice variants	No	No	Yes (D_2S, D_2L)	Yes	Yes
Affinity for DA (μm)	1.0-5.0	0.2-2.0	0.2-2.0	0.02-0.2	0.01-0.1
G protein coupling	$G\alpha_s$, $G\alpha_{olf}$	$G\alpha_s$, $G\alpha_q$	$G\alpha_i$, $G\alpha_0$	$G\alpha_i$, $G\alpha_0$	$G\alpha_i$, $G\alpha_0$

The genetic differences between both groups of receptors (D_1- and D_2-like receptors) concern the presence or absence of introns in their coding sequences: whereas the D_1-like subfamily genes do not contain introns, the genes encoding for the D_2-like subfamily have several introns. In the particular case of the D_2R gene, it contains exons that allow generating two splicing variants: long and short variants (D_2S and D_2L). These two alternatively spliced isoforms differ in the presence of an additional 29 amino acids in the third intracellular loop (Khan et al., 1998) and have distinct expression levels. For example, the D_2L mRNA is expressed at higher levels than the shorter variant (Usiello et al., 2000), and depending on the isoform (D_2S or D_2L), can be expressed presynaptically and involved in autoreceptor functions (D_2S) or predominantly postsynaptic (D_2L) (**Table 2**) (Beaulieu and Gainetdinov, 2011) .

Signalling

D_1- and D_2-like receptor subfamilies differ functionally concerning the intracellular signalling pathways. Although all DA receptors activate heterotrimeric G proteins, the actions of the second messenger pathways and effector proteins activated by both receptors are opposing. It is

commonly accepted that the D_1-like dopamine receptors (D_1 and D_5) activate the Gs/olf family of G proteins to stimulate cAMP production by adenylyl cyclase (AC) enzymes. In contrast, the D_2-like dopamine receptors (D_2, D_3, and D_4) couple to the Gi/o family of G proteins and thus induce inhibition of AC enzymes. Without a ligand agonist, the α-subunit, which contains the guanine nucleotide-binding site, is bound to guanosine diphosphate nucleotide (GDP) and a tightly associated βγ-complex to form an inactive trimeric protein complex. Upon agonist binding (DA), a sequence of events results in GDP release, GTP binding to the α-subunit and the dissociation of the α-subunit from the βγ-complex. Both the α-subunit and the βγ-complex can then transduce the signal to activate a relatively small number of effector systems. For example, the activation of Gαs proteins stimulates AC, whereas the activation of Gαi inhibits cAMP production. The cAMP formation triggers a signalling cascade by activating protein kinase A (PKA) (Pierce, Premont and Lefkowitz, 2002; Beaulieu and Gainetdinov, 2011). PKA has a variety of substrates that mediate the effects of DA receptor activation, including voltage-gated potassium (K^+), sodium (Na+), and calcium (Ca^{2+}) channels, ionotropic glutamate, GABA receptors, and transcription factors. One of the major targets of PKA is the DA and cAMP-regulated phosphoprotein (DARPP-32), a multifunctional protein highly expressed in the striatum and cortical areas that modulate downstream signal transduction pathways in response to multiple neurotransmitters, including DA. DARPP-32 increases PKA signalling by inhibiting protein phosphatase 1, which counteracts PKA's effects when PKA phosphorylates it in response to D_1R activation. Oppositely, DARPP-32 is converted into a potent inhibitor of PKA signalling when it is dephosphorylated by the calmodulin-dependent protein phosphatase 2B

in response to D_2R activation (**Figure 15**) (Beaulieu and Gainetdinov, 2011; Tritsch and Sabatini, 2012).

In addition to cAMPA/PKA-regulated signalling, DA receptors can modulate intracellular Ca^{2+} levels by acting on ion channels. After D_2R activation, βγ-subunits specifically activate phospholipase C (PLC). PLC activation results in the formation of inositol triphosphate (IP3), diacylglycerol and enhanced intracellular Ca^{2+} mobilization in response to IP3. D_2R-subunits are also involved in the control of N-type Ca^{2+} channels and G protein-coupled inwardly rectifying potassium channels (GIRKs), resulting in a suppressive impact in neurons (**Figure 15**) (Lüscher and Slesinger, 2010). Moreover, DA receptors can also engage in G protein-independent signalling events. D_1R and D_2R can alter membrane trafficking of Ca^{2+} channels, N-methyl-D-aspartate (NMDA) and $GABA_A$ receptors through direct protein-protein interactions or downstream of tyrosine kinase activation (Beaulieu and Gainetdinov, 2011).

DA receptors act through diverse signalling mechanisms, but they can also experience desensitization in response to extensive exposure to agonists and can undergo resensitization when an agonist does not activate them for a prolonged period. The phosphorylation of G protein-coupled receptor kinases produces desensitization, which recruits arrestins promoting the receptor internalization from the cellular membrane due to its binding to clathrin (Laporte et al., 2002).

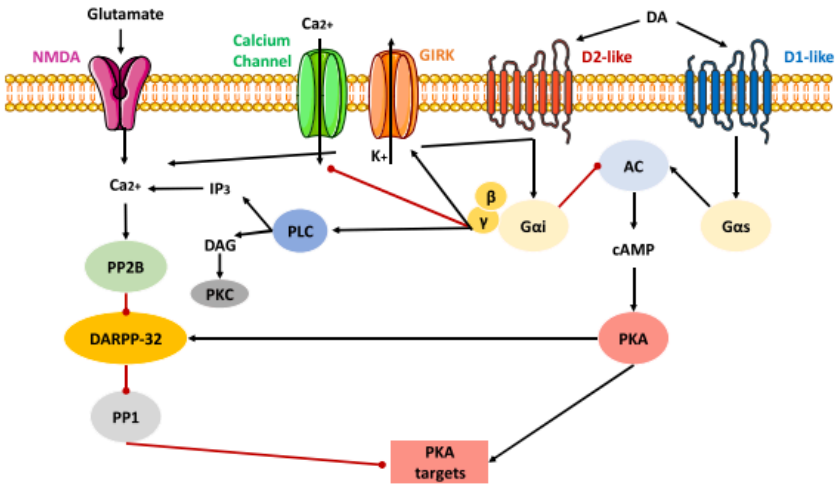


Figure 15. Intracellular DA signalling pathways. Schematic signalling networks cAMP/PKA-dependent and Gβγ-dependent regulated by D_1- and D_2-like receptor responding neurons. Black and red arrows depict activation and inhibition, respectively. NMDA, N-methyl-D-aspartate; PP2B, protein phosphatase 2B; DARPP-32, DA and cAMP-regulated phosphoprotein; PP1, protein phosphatase 1; IP3, inositol triphosphate; DAG, diacylglycerol, PLC, phospholipase C; GIRK, G protein-coupled inwardly rectifying potassium channels; AC, adenylyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase.

Distribution

Regarding receptor distribution, D_1-like receptors are found exclusively postsynaptically on dopamine receptive cells, such as GABAergic MSNs in the striatum. In contrast, the D_2-like receptors are expressed postsynaptically on dopamine target cells and presynaptically on dopaminergic neurons (Sokoloff et al., 2006; Rankin et al., 2010; Rondou, Haegeman and van Craenenbroeck, 2010). Presynaptically localized autoreceptors generally provide an important negative feedback mechanism that adjusts neuronal firing rate, synthesis, and release of the neurotransmitter in response to changes in extracellular neurotransmitter levels (Wolf and Roth, 1990; Missale et al., 1998; Sibley, 1999). Because

D2-like autoreceptors are generally activated by a lower concentration of dopamine agonists than necessary to activate postsynaptic receptors, the same dopamine agonist can induce a biphasic effect, leading to decreased activity at low doses and behavioural activation at high doses.

Although the DA receptors are widely expressed in the CNS, the most abundant subtype receptors are D_1 and D_2, being the D_1R the most widespread with the highest expression levels. Both receptors are most prominently found in areas where DA fibres innervate, being dorsal striatum, NAc and olfactory tubercle the principal recipient structures of the midbrain, but they are also presented in the cortex. The highest levels of D_2R are found in the striatum, the nucleus accumbens, and the olfactory tubercle.

2.3.2.3 Dopaminergic system in binge-eating disorder and substance of abuse

Research has revealed an important overlap between the neurobiological substrates that drive drug- and food-seeking (Schultz, 1998; Stuber, Wightman and Carelli, 2005). For example, the reward effects of drugs and some types of foods (palatable food with high energy content) implicate neuroadaptations in the dopamine (DA) system, modulating the reinforcing effects of drugs and food.

It is known that drugs of abuse generate, hijack, and amplify the dopamine reward signal and induce exaggerated and uncontrolled dopamine effects on neuronal plasticity that could develop in drug addiction. However, certain foods, particularly those rich in sugars and fat, are potent rewards (Lenoir et al., 2007) that trigger learned associations between the stimulus and the reward (conditioning) similar to drugs. This

fact was demonstrated in human studies using positron emission tomography (PET) and [^{11}C]raclopride (selective antagonist on D_2 dopamine receptors) where visual and olfactory exposure to palatable food increased extracellular DA in the dorsal striatum in normo-weight healthy controls with food deprivation for 16 hours (Volkow et al., 2002). In this study, the dopamine releases were significantly correlated with the increased in self-reports of hunger and desire for food, which provided evidence of a striatal DA release as a conditioned-cue response. These imaging studies are consistent with dopamine's role in regulating high palatable food consumption by modulating the rewarding properties of food and the motivation and desire for food consumption (Bello and Hajnal, 2010). On the other hand, preclinical studies using rodent models demonstrated that the intermittent access to sugar generates an increased dopamine release in the NAc during sugar intake similar to the reported for certain drugs of abuse. This massive dopamine release drives to consumption of abnormal amounts of sugar in a binge-like manner similar to that observed in animal models of drug dependence (Avena, Rada and Hoebel, 2009). The increased DA release during high palatable food in striatal areas partly explains in part the behavioural similarities observed between patients diagnosed with binge-eating disorder and substance abusers in increased compulsive and impulsive behaviours towards the reinforcer (Wang et al., 2004; Galanti, Gluck and Geliebter, 2007).

It has been postulated that in humans, low dopamine activity could predispose a person to pathological overeating, as a way to compensate for decreased dopaminergic activity (rewarding effects) (Blum et al., 1996). Specifically, dysregulations in dopaminergic (DA)

transmission and changes in the activity of the D_2R in the striatum seem to play a relevant role in appetitive regulation and compulsive motivation to consume palatable food beyond homeostatic needs (Volkow et al., 2011; Wang et al., 2011b; Tomasi and Volkow, 2013; Volkow and Baler, 2015). Indeed, neuroadaptations in the reward system (decreased striatal D_2R) have been found in obese and drug-dependent individuals (Volkow, Wang, Telang, et al., 2008b; Volkow et al., 2011). Furthermore, abnormal dopaminergic activity has also been demonstrated in genetically inbred rodents for obesity and has been postulated to underlie overeating (Thanos et al., 2008). Thus, dopamine modulates motivation and reward circuits and hence dopamine deficiency in obese subjects may perpetuate pathologic eating to compensate for decreased activation of these circuits. However, recent studies comparing obese with and without binge-eating disorder revealed a lack of correlation between BMI and DA changes, suggesting that DA release *per se* does not predict BMI but predicts binge eating (Wang et al., 2011b). In addition to this, other studies support the correlation between functional markers of the D2R, rs1800497 and rs6277, with the binge-eating disorder than weight-matched controls and a decreased probability of carrying the minor T allele rs2283265 (Davis et al., 2012). Altogether, highlight the DA motive system, specifically the D_2R, in regulating the mesolimbic pathway as interesting targets to investigate the development of binge-eating episodes and the influence of other components of the reward system in its activity regulation.

2.3.3 Endocannabinoid signalling

The endocannabinoid system (ECS) is a neuromodulatory system that plays several physiological roles, including reward-related processes. It is

widely distributed in the central and peripheral nervous system (Katona and Freund, 2012) and in many other tissues (Piazza, Cota and Marsicano, 2017), where it regulates brain functions by acting on different cell types and cellular compartments (Katona and Freund, 2012; Piazza, Cota and Marsicano, 2017; Busquets-Garcia et al., 2018). This system is integrated by the endogenous cannabinoid receptors, endogenous ligands (endocannabinoids, eCBs), and the enzymes involved in their synthesis and degradation, as well as intracellular signalling pathways and transport systems (Piomelli, 2003; Katona and Freund, 2012; Lutz et al., 2015; Pertwee, 2015).

2.3.3.1 Cannabinoid receptors: CB_1 and CB_2 in the central nervous system

The two canonical receptors of the endocannabinoid system are the cannabinoid receptor 1 (CB_1R) and 2 (CB_2R), which are G protein-coupled receptors (GPCRs) with seven transmembrane domains (Childers and Deadwyler, 1996). The **CB_1R** is highly expressed in the central nervous system (CNS), being the most abundant G protein-coupled receptor in the brain. Its expression is extensive and has been well characterized in rodents (K Tsou et al., 1998) and humans (Westlake et al., 1994). The CB_1R plays a critical role in regulating different brain functions and in pathological processes (K. Tsou et al., 1998; Marsicano et al., 2002; Soria-Gómez et al., 2014; Martín-García et al., 2016; Gutiérrez-Rodríguez et al., 2018), including addictive-related behaviours. In addition, several areas belong to the mesocorticolimbic circuit present high levels of expression of CB_1R, such as the basal ganglia (substantia nigra pars reticulata, globus pallidus, striatum, entopeduncular nucleus), cortex, NAc, and

hippocampus (Howlett et al., 1990; K. Tsou et al., 1998; Shu-Jung Hu and Mackie, 2015; Martín-García et al., 2016). Despite its broad expression in the CNS, studies using conditional mutant mice lacking CB_1R suggest that this receptor is primarily found in GABAergic neurons (Monory et al., 2006; Marsicano and Kuner, 2008; Martín-García et al., 2016). In particular, CB_1R expression is very high in inhibitory GABAergic synaptic terminals mostly in cortical and hippocampal cholecystokinin (CCK)-positive GABAergic interneurons (Kawamura et al., 2006; Marsicano and Kuner, 2008; Katona and Freund, 2012; Steindel et al., 2013; Shu-Jung Hu and Mackie, 2015), low in excitatory glutamatergic synapses (Marsicano et al., 2003; Monory et al., 2006; Puente et al., 2011; Ruehle et al., 2013; Gutiérrez-Rodríguez et al., 2017) and very low in brain astrocytes (Rodríguez, Mackie and Pickel, 2001; Metna-Laurent and Marsicano, 2015; Gutiérrez-Rodríguez et al., 2018). CB_1R is primarily found in axon terminals or in close proximity to the presynaptic active zones in the brain, although they are also found in mitochondria in neurons (Bénard et al., 2012; Hebert-Chatelain et al., 2014a, 2014b; Koch et al., 2015) and astrocytes [33]. Thus, CB_1R is distributed in GABAergic terminals (∼56%), glutamatergic terminals (∼12%), astrocytes (∼6%), mitochondria (∼15%) and other compartments (∼11%) in the hippocampus (**Figure 16**) (Gutiérrez-Rodríguez et al., 2018; Bonilla-Del Río et al., 2019).

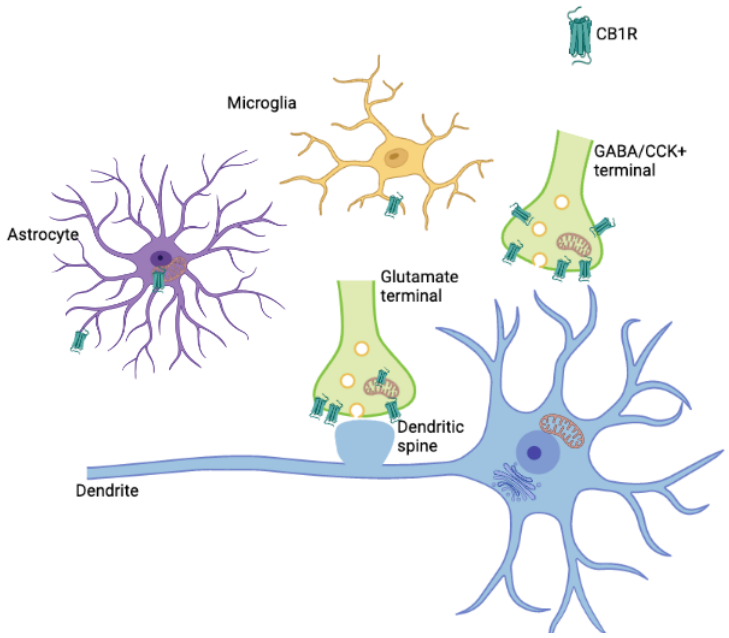


Figure 16. Distribution of CB_1R in the CNS. Adapted from (Manzanares et al., 2018)

In contrast, CB_2R was initially considered the peripheral cannabinoid receptor due to its high expression in the rat spleen (Munro, Thomas and Abu-Shaar, 1993) and leukocyte subpopulation in humans (Sylvaine Galiègue et al., 1995). This receptor is mainly expressed in the periphery, particularly in immune cells like macrophages, B and T lymphocytes, neutrophils and monocytes (S Galiègue et al., 1995). Curiously, the CB_2R was not identified in CNS under basal conditions until 2005 (van Sickle et al., 2005), being only detected until then under pathological conditions such as in senile plaques in Alzheimer's disease (Benito et al., 2003), activated microglial cells/macrophages in multiple sclerosis, spinal cord in amyotrophic lateral sclerosis (Yiangou et al., 2006) and in the vicinity of tumours (Guzmán, Sánchez and Galve-Roperh, 2001). However, nowadays, several authors support the idea that CB_2R is expressed in

neurons of the brainstem of mice, rats and ferrets under normal conditions (van Sickle et al., 2005), as well as in cortical, hippocampal, pallidal and mesencephalic neurons (Lanciego et al., 2011), and in the hippocampus, frontal cortex, amygdala and striatum (Gong et al., 2006; Onaivi et al., 2006; Onaivi, 2007; Brusco et al., 2008; Onaivi, Ishiguro, Gong, Patel, Meozzi, Myers, Perchuk, Mora, Tagliaferro, Gardner, Brusco, Akinshola, Hope, et al., 2008; Onaivi, Ishiguro, Gong, Patel, Meozzi, Myers, Perchuk, Mora, Tagliaferro, Gardner, Brusco, Akinshola, Liu, et al., 2008).

Cannabinoid receptors exert their actions by activating heterotrimeric Gi/o proteins (**Figure 17**) (McAllister and Glass, 2002). When Gi/o proteins are activated, adenylyl cyclase activity (AC) is inhibited, resulting in a decrease in cyclic adenosine monophosphate (cAMP) synthesis and a decrease in protein kinase A (PKA) activity. Coupling to heterotrimeric Gi/o proteins also stimulates MAPK family enzymes such extracellular signal-regulated kinases 1 and 2 (ERK1/2), p38, and c-Jun N-terminal kinase (JNK) (Bosier et al., 2010). Furthermore, activation of complex protein cascades, including those involving phosphoinositide-3-kinase (PI3K), has been hypothesized (Piomelli, 2003). Gene transcription is regulated downstream as a result of these intracellular processes. In neurons, activating linked Gi/o proteins inhibits neurotransmitter release by inhibiting voltage-activated Ca2+ channels and stimulating inwardly rectifying K+ channels (Kir3) (McAllister and Glass, 2002).

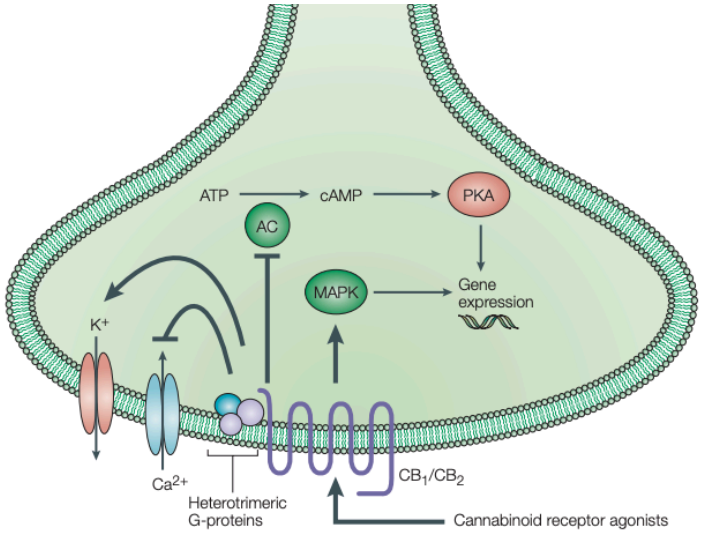


Figure 17. Major signalling pathways of cannabinoids. CB_1, cannabinoid receptor 1; CB_2, cannabinoid receptor 2; AC, adenylyl cyclase; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; MAPK, mitogen-activated protein kinase. Adapted from (Di Marzo, Bifulco and De Petrocellis, 2004).

2.3.3.2 Endocannabinoid ligands in the central nervous system

The eCBs are lipid messengers (long-chain arachidonic acid derivatives) that can diffuse and traverse membranes in a paracrine, autocrine, and possibly endocrine manner (Piomelli, 2003; Kano et al., 2009; Katona and Freund, 2012; Lu and MacKie, 2016; Zou and Kumar, 2018). There are two main endocannabinoids, N-arachidonoylethanolamine (anandamide or **AEA**) and 2-arachidonoylglycerol (**2-AG**) (Devane et al., 1992; Mechoulam et al., 1995). The different endocannabinoids exhibit varying selectivity for CB1R and CB2R and other targets (McAllister and Glass, 2002), as well as different concentration distribution. For example, **AEA** is a partial agonist of CB_1R and CB_2R and also has an affinity for TRPV1 (Cristino et al., 2008),

while **2-AG** is a full agonist of CB1R and CB2R, and it is present at higher concentrations than AEA in the brain (Sugiura et al., 2006).

The balance concentration of the eCBs is regulated by enzymatic synthesis and degradation (**Figure 18**). **AEA** synthesis involves two enzymatic reactions. The AEA precursor N-arachidonoyl phosphatidylethanolamine (NAPE) is produced by the Ca2+ dependent N-acyltransferase transferring arachidonic acid from phosphatidylcholine to phosphatidylethanolamine (Cadas et al., 1996; Kano et al., 2009; Fezza et al., 2014). Then, AEA synthesis is produced by the N-acylphosphatidylethanolamine specific phospholipase D (NAPE-PLD) that hydrolyses N-arachidonoyl phosphatidylethanolamine localized in cell membranes (Okamoto et al., 2004; Kano et al., 2009). Once AEA is synthesized, its half-life is very short. The fatty acid amide hydrolase (FAAH) is a serine-hydrolase enzyme present in the brain and many organs (Dinh et al., 2002; Kano et al., 2009). This enzyme is responsible for AEA inactivation that catalyzes AEA into arachidonic acid and ethanolamine (Fezza et al., 2014) at the postsynaptic level. Moreover, the AEA half-life is short because of its quick re-uptake by a high-affinity AEA membrane transporter distributed in neurons and glia (di Marzo, Stella and Zimmer, 2015). **2-AG**, on the other hand, participates in the CB_1R-dependent retrograde signalling and is synthesized followed neuronal membrane depolarization. First, the diacylglycerol precursors come from the hydrolysis of membrane phosphatidylinositol by phospholipase C, β or δ, then the degradation of these precursors by DAGL-α and DAGL-β leads to 2- AG synthesis (Kano et al., 2009; Gao et al., 2010; Lu and MacKie, 2016; Zou and Kumar, 2018). The DAGL-α isoform synthesizes the most 2-AG, while the DAGL-β isoform synthesizes 2-AG under certain conditions (di

Marzo, Stella and Zimmer, 2015). 2-AG is primarily metabolized by monoacylglycerol lipase (MAGL or MGLL) (Dinh, Freund and Piomelli, 2002), a serine-hydrolase enzyme mainly found in presynaptic terminals (Dinh et al., 2002; Kano et al., 2009; Shu-Jung Hu and Mackie, 2015; Lu and MacKie, 2016).

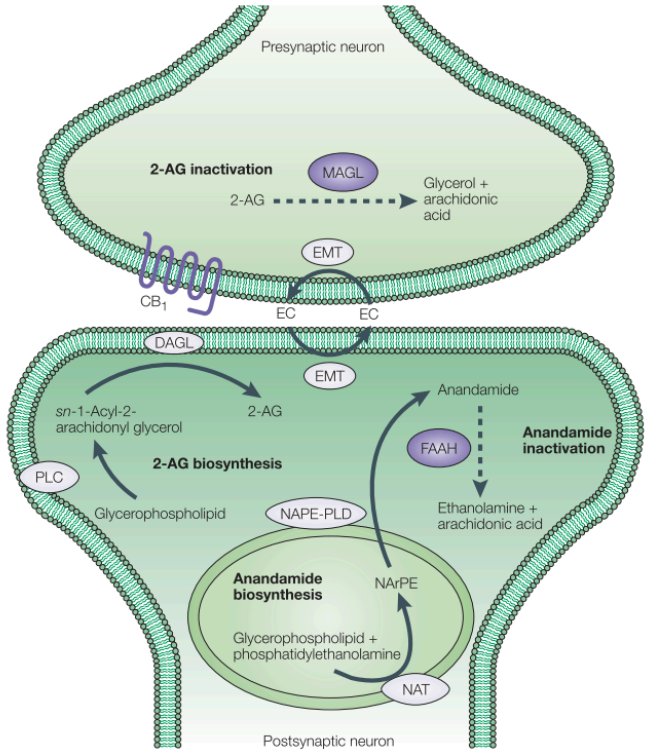


Figure 18. Synthesis and degradation of endocannabinoids. CB_1, cannabinoid receptor 1; EC, endocannabinoid; 2-AG, 2-arachidonoylglycerol; PLC, phospholipases C; DAGL, diacylglycerol lipase; MAGL, monoacylglycerol lipase; NAT, N-acyltransferase; NAPE-PLD, N-acylphosphatidyl-ethanolamine specific phospholipase D; FAAH, fatty acid amide hydrolase; EMT, endocannabinoid membrane transporter; NArPE, N-arachidonoyl-phosphatidyl-ethanolamine. Adapted from (Di Marzo, Bifulco and De Petrocellis, 2004).

Unlike other neurotransmitters, endocannabinoids are a family of molecules that are not pre-stored in secretory vesicles but are synthesized

on-demand from cell membrane lipids and released in response to an increase in intracellular calcium concentration. Another characteristic is that they act as retrograde messengers (Freund, Katona and Piomelli, 2003) over presynaptic neurons (**Figure 18**). The activation of presynaptic CB1R leads to the suppression of neurotransmitter release on excitatory or inhibitory synapses. This neurotransmitter suppression may result in short-term or long-term plasticity. In this regard, the CB1R activation for a few seconds results in short-term plasticity with transient depolarization-induced suppression of inhibition or excitation (depending on the type of the presynaptic terminal) (Castillo et al., 2012). However, the long-term plasticity depression at both excitatory and inhibitory synapses is mediated by the inhibition of AC and downregulation of the cAMP/PKA pathway in active synapses (Heifets and Castillo, 2009).

2.3.3.3 Endocannabinoid modulation of brain's reward system

As explained above, one of the principal substrates of the rewarding effect is the mesocorticolimbic pathway (see section 2.2). The CB_1R is present in the main structures of the mesocorticolimbic system with a strong influence in the modulation of excitatory and inhibitory signalling, affecting reward control processing and food intake (Parsons and Hurd, 2015). The expression of CB_1R is medium-high in the dorsal and ventral striatum and low in the VTA (Herkenham et al., 1990; K. Tsou et al., 1998; Martín-García et al., 2016). However, the DA efflux from the VTA projections is regulated by glutamatergic projecting neurons to the VTA and GABAergic interneurons expressing CB_1R at the presynaptic level. Postsynaptic eCBs act retrogradely on presynaptic CB_1R, modulating the activity of GABAergic or glutamatergic neurons. Thus, the GABAergic

transmission that inhibits VTA DA neurons can be inhibited (depolarization-induced suppression of inhibition, DSI) by activating the CB_1R at the presynaptic level (D'Addario et al., 2014), improving disinhibition and promoting reward by increasing the DA release in the VTA-NAc pathway. On the contrary, glutamatergic signalling that activates VTA DA neurons and induces long-term potentiation associated with the hedonic responses (Maldonado, Valverde and Berrendero, 2006) can be inhibited by postsynaptic eCBs acting retrogradely on presynaptic CB_1R that decrease excitatory glutamatergic transmission (depolarization-induced suppression of excitation, DSE) in the VTA to NAc (**Figure 19**) (Melis et al., 2004).

The NAc neurons activity is modulated by the low to moderate CB_1R levels and abundant DA receptors (Hernandez, Sadeghian and Kelley, 2002; Mato et al., 2005; Salamone et al., 2007). Concerning the role of the ECS in this area, the CB_1Rs are localized at a postsynaptic level in excitatory projections from PFC to NAc (glutamatergic) and at a presynaptic level in inhibitory projection from NAc (GABAergiC) in the medium-spiny neurons (MSNs) and parvalbumin-positive interneurons (**Figure 19**). Thus, the rewarding effects of different substances are also modulated by the eCBs acting directly into the NAc. Local microinjection of CB_1R antagonist into NAc attenuates ethanol responding by reducing the GABA release from the projecting MSNs. Similarly occurs for the hedonic effect of palatable food suppressing the saccharin intake in rodents following local injection of CB_1R antagonist into the NAc (Malinen and Hyytiä, 2008). But the NAc neurons activity is also modulated from the endocannabinoids acting via CB_1R on glutamatergic terminals in the NAc, reducing the glutamate release and inducing hyperphagia (Bellocchio et al., 2010). Therefore, the

final effect of endocannabinoids on food intake depends on the functional balance between its actions on inhibitory GABAergic versus excitatory glutamatergic transmission.

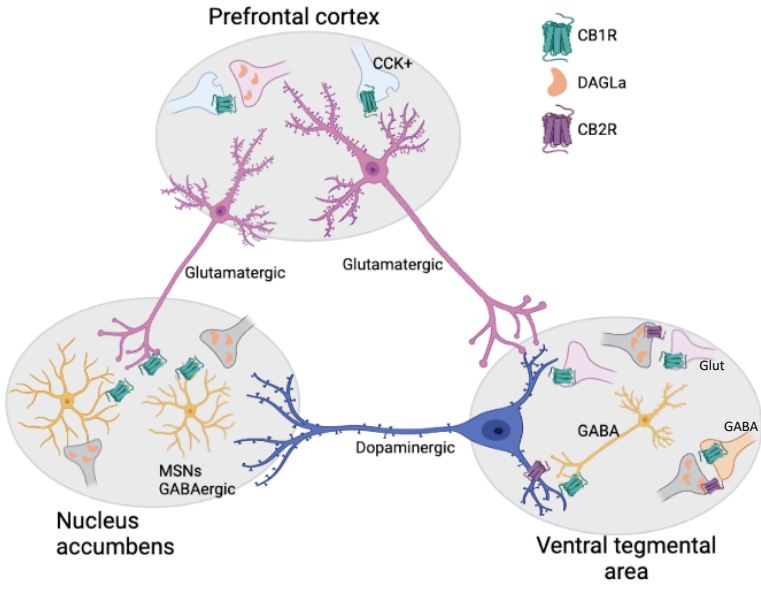


Figure 19. Functional localization of CB1R and CB2R in the main structures in the mesocorticolimbic circuit. CCK+: Cholecystokinin-positive. Adapted from (Manzanares et al., 2018)

The endocannabinoid system has another important role in controlling food intake through sensory perception such as palatability and olfaction, increasing food intake. Local pharmacological and genetic manipulations revealed that cortical feedback projection to the main olfactory bulb crucially regulates food intake via CB_1R (Soria-Gómez et al., 2014). These results demonstrate that the endocannabinoids of the mesocorticolimbic system play a prominent role in the reinforcing properties of drugs and food intake by its neuronal activity regulation function (Busquets-Garcia

et al., 2015), being an important target of research for the hedonic effect evoked from palatable food.

2.3.3.4 The endocannabinoid system in eating disorders

The widespread role of the endocannabinoid system as a modulator of both homeostatic and hedonic aspects of food intake prompted investigations into possible alterations of this system in eating disorders and obesity. For example, women diagnosed with anorexia nervosa or binge-eating disorder presented enhanced levels of AEA compared to the control group, suggesting a possible involvement of AEA in the mediation of the rewarding aspects of the aberrant eating behaviours occurring in anorexia nervosa and binge-eating disorder (Monteleone et al., 2005). According to these results, PET studies revealed increased CB_1R availability in cortical and subcortical brain areas in anorexia nervosa patients compared with healthy control (Gérard et al., 2011). Furthermore, genetic studies in humans also revealed specific polymorphisms of genes encoding for different components of the endocannabinoid system, such as CB_1R and fatty acid amide hydrolase (FAAH), significantly associated with anorexia and bulimia nervosa (Monteleone et al., 2009). Based on these findings, it has been hypothesized that the dysregulated endocannabinoid tone of eating disorders patients may represent an adaptative response to maintain energy balance by potentiating internal orexigenic signals and facilitating the rewarding properties of food intake (Monteleone and Maj, 2013; D'Addario et al., 2014).

Several preclinical and clinical observations have shown associations between obesity and hyperactivity of the ECS manifested as

overproduction of eCBs or/and upregulation of cannabinoid receptors in central and peripheral tissues involved in energy homeostasis (di Marzo and Matias, 2005). These findings indicate an important role of the ECS in regulating the hedonic aspects of feeding. A recent study using a validated food addiction mouse model performed in our laboratory found that long-term operant training to obtain highly palatable food produced adaptative changes at epigenetic and protein levels in the endocannabinoid system (Mancino et al., 2015). Specifically, we observed a significant reduction in DNA methylation at CB_1R gene (Cnr1) promoter in PFC, which was associated with upregulation of gene expression and the subsequent increase in CB1R protein in mice classified as food addicted. The involvement of the CB_1R in the food addictive-like behaviour was corroborated using pharmacological and genetic approaches. Administration of a CB_1R antagonist reduced the percentage of animals that reached the addiction scores. In accordance, the genetic deletion of the CB_1R (CB1KO mice) decreased operant seeking behaviour, and these KO mice did not reach the criteria for addiction (Mancino et al., 2015). These results were further investigated in recent studies where Glu-CB_1-KO mice displayed a resilient phenotype to develop food addiction-like behaviour, which was linked to enhanced excitatory synaptic transmission of glutamatergic neurons in the PL-NAc pathway (Domingo-Rodriguez et al., 2020). Based on these results, we hypothesized that the ECS plays an important role in regulating the overconsumption of high-fat content food and obesity (Ruiz de Azua et al., 2021) through the modulation of the hedonic effects evoked by the high palatability of this type of diet.

3 Other neurotransmitters

Although compulsive overeating (such as binge eating episodes) is an aberrant feeding behaviour modulated by interconnected neuronal networks that involve both homeostatic and allostatic regulation, the DA reward system (see section 2.3.2) in the mesolimbic circuit plays a pivotal role to understand such maladaptive habits formations. However, other neurotransmitters may regulate the DA release in the NAc and the GABA release from NAc to other brain areas in the brain's reward system.

3.1 Cholecystokinin in food intake regulation

Cholecystokinin (CCK) consists of a family of peptides, but the best characterized is a 33 amino acid peptide secreted from endocrine cells in the jejunum in response to nutrients in the intestinal lumen. This gut-derived peptide has several gastrointestinal functions, including promoting satiation/satiety (Gibbs, Young and Smith, 1973; Saito, Williams and Goldfine, 1981; Smith and Gibbs, 1994) (see section 2.1.1.1). This peripherally derived CCK does not readily penetrate the brain (Passaro et al., 1982; Fan et al., 1997) but can stimulate the vagal sensory neurons influencing the brainstem (Gibbs, Young and Smith, 1973; Saito, Williams and Goldfine, 1981; Smith and Gibbs, 1994; Fan et al., 1997). Nevertheless, CCK is also synthesized within the brain, where it is post-translationally processed into an 8 amino acid peptide (CCK-8). This neuropeptide is implicated in centrally food intake regulation (Hirosue et al., 1993; Blevins et al., 2000) and DA-mediated behaviours responses (White and Wang, 1984; Crawley, 1988; Vaccarino, 1994; Lança et al., 1998), among others.

3.1.1 Central regulation

CCK-8 is the most abundant neuropeptide found in the brain (Crawley and Corwin, 1994) and exerts complex functional interplays with **DA** (Höckfelt, T., Rehfeld, J.F., Skirbol, L.L., 1980; Rotzinger, Bush and Vaccarino, 2002), **opioids** (Hebb et al., 2005), **GABA** (Freund and Katona, 2007), **serotonin** or **5-HT** (Stallone, Nicolaidis and Gibbs, 1989; Cooper, Dourish and Barber, 1990; Grignaschi et al., 1993; Morales and Bloom, 1997; Férézou et al., 2002) and **glutamate** (Nguyen et al., 2020). There are two CCK receptors subtypes, CCK_A and CCK_B, widely distributed in the brain. Among the **roles** attributed to the activation of CCK receptors are the involvement in **reward-related behaviours** (Vaccarino, 1994; Rotzinger and Vaccarino, 2003), **anxiety** (van Megen et al., 1996; Koszycki et al., 2012) and **satiety**, being all these attributes important targets for treating eating disorders, particularly binge-eating disorder.

Concerning **reward-related behaviour**, CCK is abundant in the NAc and colocalized with DA in afferent terminals in this region from the VTA (Pernow B, 1980) and substantia nigra (SN) (Lança et al., 1998) modulating accumbal DA turnover (Dumbrille-Ross and Seeman, 1984; Vaccarino, 1994) to regulate **stress-motivated behaviours** (Rotzinger, Bush and Vaccarino, 2002). The effects of CCK on DA-mediated behavioural responses have been seen to vary along the medio-lateral (White, F. J.; Wang, 1986) and rostro-caudal axis of the NAc (Crawley, Hommer and Skirboll, 1985; de Witte et al., 1987; Kariya, Tanaka and Nomura, 1994). Thus, it has been found that injections of the sulfated form of CCK-8 into the medio-caudal part of the NAc induced a strong increase of intracranial self-stimulation of the medial forebrain bundle, while a similar administration in the rostral NAc caused a moderate decrease (de Witte

et al., 1987). Further, injections of the non-selective CCK receptor antagonist proglumide in the caudal and rostral NAc produced attenuation and potentiation, respectively, of the intracranial self-stimulation in the ventral tegmental area (Vaccarino and Vaccarino, 1989). The behavioural data indicate that different actions of CCK on dopamine function depend on the accumbal area studied, consistent with various neurochemical studies using brain slices. For instance, CCK has been found to increase DA resting release from the posterior but not anterior NAc (Voigt, Wang and Westfall, 1986). CCK potentiates K^+-stimulated release in the posterior NAc, whereas inhibited K^+-stimulated DA release in the anterior NAc (Vickroy and Bianchi, 1989; Marshall et al., 1991). Both actions (i.e., potentiation and inhibition of the K^+-stimulated release of DA) were blocked by a CCK_A receptor antagonist (devazepide) in the posterior NAc and a CCK_B receptor antagonist (L365,260) in the anterior NAc, respectively (Marshall et al., 1991). This supports the view that the opposite effects of CCK on DA activity seen along the rostrocaudal axis of the NAc are associated with actions on different receptor subtypes.

In addition to the CCK/DA system modulation, the serotonergic system is also implicated in the CCK-mediated modulation of rewarding effects. 5-HT can act as a potent releaser of CCK in rat NAc through activating receptors of the 5-HT_3 type situated on CCK-releasing terminals (Paudice and Raiteri, 1991). Moreover, 5-HT_3 receptors are also expressed in a high proportion of GABAergic neurons containing CCK immunoreactivity in the telencephalon (Morales and Bloom, 1997), which mediate fast synaptic excitation CCKergic interneurons by serotonergic afferent fibres from the brainstem raphe nuclei (Férézou et al., 2002). It follows that 5-HT, through the activity of 5-HT_3 receptors, may act

synergistically with CCK release in the NAc and the cerebral cortex (Raiteri, Paudice and Vallebuona, 1993) to modulate motivation (Vasar et al., 1993).

However, eating disorders, in particular binge-eating disorders, often co-occur with other psychiatric disorders, especially mood and **anxiety disorders** and obsessive-compulsive disorders. This comorbidity highlights the interconnected neuronal network and the close relationship between different brain areas and pathways. As mentioned above, CCK also has a behavioural role in **anxiety**. In this sense, as is described for alcohol (Breese et al., 2005), excessive consumption is often linked to anxiety states. Voluntary alcohol and food intake-stress-anxiety interactions have a major relay in the extended amygdala. CCKergic neurons in this brain region are implicated in the modulation of emotional states (Andrés et al., 1993; Giardino et al., 2018). Particularly, CCK_B receptors mediate stress-induced drug craving (Lu et al., 2002), which may be correlated with food craving.

In addition to the role of CCK in reward-related behaviour and emotional states modulation, CCK in the brain can also act as a **satiety signal**. It has long been known that alcohol and food intake circuits overlap (Thiele et al., 2004). For example, CCK can act as a satiety signal for both food (Gibbs, Young and Smith, 1973; Moran and Kinzig, 2004) and ethanol (Geary et al., 2004), and as with feeding behaviour, the spontaneous preference for ethanol is influenced by CCK_A receptors (Crespi et al., 1997). This CCK subtype receptor stimulates oxytocin secretion from magnocellular neurons of the supraoptic nucleus in the posterior pituitary gland (Hashimoto et al., 2005). Oxytocin serves as a neuromodulator of the human autonomic nervous system, especially in vagal pathways

(Carter, 2014) that **affect mood well-being** (Ishak, Kahloon and Fakhry, 2011), but also have a physiological role in **food intake** and **energy balance** through inhibition of the **reward pathway** (Sabatier, Leng and Menzies, 2013). Thus, it was hypothesized that the reduction of alcohol preference, craving, withdrawal symptoms, and cue-reactivity attributed to oxytocin (Bach et al., 2019; Tunstall et al., 2019; King, Gano and Becker, 2020) could be due to the interactions with CCK within the **stress** and **addiction neurocircuitry**, resulting in an increased sense of well-being and a reduction of anxiety triggered by abstinence from alcohol. Given the CCK involvement in different aspects in regulating alcohol intake (reward-related behaviour, emotional states and satiety), CCK has emerged as a promising target for eating disorder research that presents significant overlapping with substances abuse disorders.

3.2 Adenosine in food intake regulation

Adenosine is an endogenous modulator released in the CNS and periphery that acts on distinct cell-surface receptors coupled to G-proteins, which are termed A_1, A_{2A}, A_{2B}, and A_3 receptors (Fredholm et al., 1994). A_1R and A_3R are mainly coupled to the inhibitory G_i or G_o protein, and A_2Ar as well as $A_{2B}R$ mostly coupled to the stimulatory G_s, G_q or G_{olf} protein (Schulte and Fredholm, 2003; Chen, Eltzschig and Fredholm, 2013). Each type of adenosine receptor has different functions, although with some overlap (Gao and Jacobson, 2011). For instance, at periphery level, both A_1 receptors and A_{2A} play roles in the heart, regulating myocardial oxygen consumption and coronary blood flow, while the A_{2A} receptor also has broader anti-inflammatory effects throughout the body (Haskó and Pacher, 2008). These two receptors also have important roles in the brain

(Chen, 2018), regulating the release of other neurotransmitters such as as dopamine and glutamate (Fuxe et al., 2007; Cunha et al., 2008; Chen, 2018), while the A_{2B} and A_3 receptors are located mainly peripherally and are involved in processes such as inflammation and immune responses. In the brain, the A_{2A} receptors are highly expressed in the dorsal and ventral striatum and in reduced but significant densities in the hippocampus and the cortex. Particularly in the NAc, the A_{2A} subtype receptor is expressed with a high density (Ongini and Fredholm, 1996; Rosin et al., 1998) and plays a critical role in behavioural control (Ferré, 1997) by forming a heteromeric complex with the dopamine type-2 receptor (D_2R). Given the extensive research on the D_2R role in regulating addictive and DA-reward behaviour, the couple interaction between these two receptors has become an important focus of research in eating disorders.

A_{2A} receptors localization in the brain

The pattern distribution of A_{2A}Rs is similar in both humans and rodents, with the highest expression reported in striatal areas (dorsal and ventral striatum) and lower levels in the hippocampus, hypothalamus, thalamus, cerebral cortex, amygdala, thalamic nuclei, and substantia nigra (Jarvis and Williams, 1989; Cunha et al., 1994; Dixon et al., 1996; Peterfreund et al., 1996; Rosin et al., 1998, 2003; Svenningsson et al., 1999; Rebola et al., 2005). Anatomical, electrophysiological, and biochemical approaches have localized this receptor on neurons and glial cells (including astrocytes and microglia) (Rosin et al., 1998, 2003; Nishizaki et al., 2002; Lee et al., 2003; Rebola et al., 2005). At the striatum, A_{2A}Rs can be localized on neurons (90%), **postsynaptically** (70%, on dendrites and dendritic spines), **presynaptically** (23%, on terminals of axon collaterals), and

extrasynaptically (3%, on somas) (Rosin et al., 1998, 2003; Rebola et al., 2005; Quiroz et al., 2009; Sebastiao and Ribeiro, 2009). At the postsynaptic level, striatal A_{2A}Rs are mainly localized to the gamma-aminobutyric acid GABAergic-MSNs of the indirect pathway colocalizing with D_2R and enkephalin (Schiffmann, Jacobs and Vanderhaeghen, 1991; Fink et al., 1992; Augood and Emson, 1994; Svenningsson et al., 1999; Rosin et al., 2003; Rebola et al., 2005; Schiffmann et al., 2007). Inversely, neurons that selectively express D_1 and the peptide dynorphin did not contain a significant level of A_{2A}Rs (Schiffmann, Jacobs and Vanderhaeghen, 1991). At the presynaptic level, A_{2A}Rs are mainly localized on cortico-thalamic glutaminergic terminals that contact GABergic-MSNs in the direct and indirect pathways (Rosin et al., 2003; Rodrigues et al., 2005; Quiroz et al., 2009) and on cholinergic neurons that modulate acetylcholine release (Brown et al., 1990; Kurokawa et al., 1994).

Using different co-localization approaches such as double immunofluorescence, co-immunoprecipitation, resonance energy transfer techniques (SRET), and in vivo proximity ligation assay (PLA), researchers have been demonstrated that most functional A_{2A}Rs form dimers with other receptors. In the striatum, they can form either homodimer (A_{2A}R-A_{2A}R) on the cell surface or heterodimers with other metabotropic GPCRs such as A_1, A_{2B}, A_3, CB_1, D_2, D_3, glutamatergic Glu type 5 ($mGlu_5$), fibroblast growth factor receptor (FGFR1), and Sigma1 receptors (**Figure 20**) (Canals et al., 2003, 2004; Sebastiao and Ribeiro, 2009; Borroto-Escuela et al., 2014, 2016, 2018).

Although A_{2A}Rs can be found forming a heteromeric complex with different receptors (such as A_{2A}-CB_1-D_2 and A_{2A}-D_2-$mGlu_5$) in the striatum, the first recognized and best characterized heteromeric

interaction was found between the A_{2A}Rs and D_2R. In this particular heterodimer complex, activating A_{2A}Rs (Gs/olf-coupled) interfered with coupling D_2Rs to Gi isoforms of G proteins (Ferre et al., 2008; Fuxe et al., 2010; Ferré et al., 2011). These A_{2A}-D_2 heteroreceptor complexes with **antagonistic receptor-receptor interaction** have been found in dorsal and ventral striatopallidal GABA as antireward pathway (Fuxe et al., 2003, 2005); on the cortico-striatal glutamate nerve terminals, inhibiting the glutamate release (Tozzi et al., 2007); on striatal cholinergic interneurons (Tozzi et al., 2011) and on striatal astrocytes (Cervetto et al., 2017, 2018).

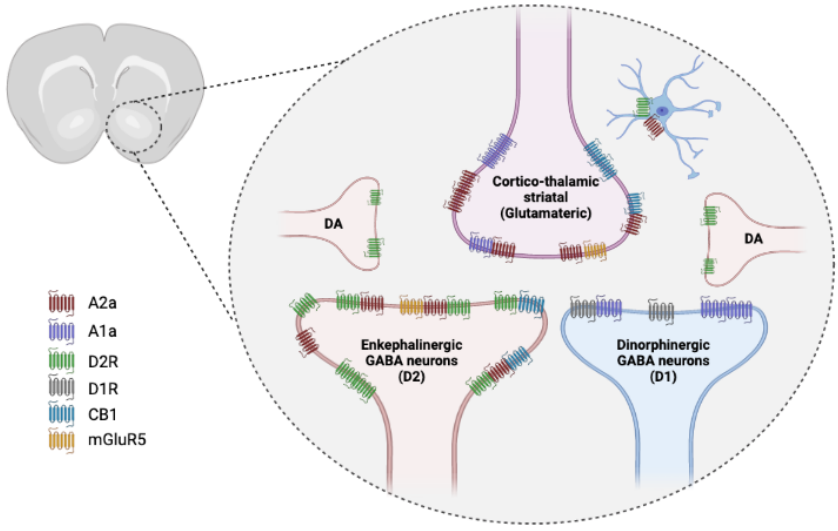


Figure 20. Schematic diagram showing the neuronal and astrocytic localization of adenosine A_{2a} homo-, heterodimer, and oligomer complexes with dopamine (D), cannabinoid (CB), and metabotropic glutamate receptors (mGlu) on glutamatergic input from the cortex and thalamus and dopaminergic (DA) input from the ventral tegmental area and both enkephalin striatopallidal and dynorphin striatonigral gamma-aminobutyric acid (GABA) neurons in the rat striatum. Cholinergic interneurons are not included. Adapted from (Wydra et al., 2020)

A_{2A}R in behavioural actions to substances of abuse and palatable food

Numerous preclinical studies confirmed functional interactions between A_{2A}Rs and drugs of abuse with different mechanisms of action on the central nervous system. Studies using traditional pharmacological tools (agonists or antagonists of A_{2A}Rs) as well as animal genetic models (overexpression or knockout (KO) of A_{2A}Rs) have shown that these receptors are involved in the **locomotor response** (Turgeon et al., 1996; Rimondini et al., 1997), **drug discrimination tasks** (Justinova et al., 2003, 2009), **reward-seeking behaviour** (Knapp et al., 2001; Wydra, Gołembiowska, et al., 2015), and **withdrawal syndrome** (Wydra, Suder, et al., 2015) associated with the use of psychostimulants (cocaine, amphetamine, methamphetamine, and 3,4-methylenedioxy methamphetamine (MDMA)), nicotine, opioids (morphine and heroin), cannabinoids (Δ9-tetrahydrocannabinol (THC)), and ethanol.

Several studies indicated the role of A_{2A}Rs in the locomotor effects of drugs of abuse. For example, hyperlocomotion induced by acute cocaine administration can be decreased by CGS 21680 (A_{2A}R agonist) injection even in well-habituated rats to cocaine (Filip et al., 2006). Similarly, studies using genetically modified rats overexpressing A_{2A}R reported that a single injection of cocaine did not increase locomotion in transgenic rats as observed in the wild-type group. Although locomotor activity response is sometimes used to indicate a propensity for psychostimulant self-administration, some authors argue that it is less about locomotor activity and more about environmental novelty and learning rate of an operant task (Mitchell, Cunningham and Mark, 2005). In this sense, NAc DA modulates the performance of goal-directed behaviours and is involved in various aspects of instrumental learning and motivation (Ikemoto and

Panksepp, 1996; Salamone and Correa, 2002; Kelley, 2004; Barbano and Cador, 2007; Berridge, 2007; Robbins and Everitt, 2007; Salamone et al., 2007). An important function of accumbens DA is the modulation of behavioural activation and effort-related processes, particularly for tasks that require substantial effort to obtain the goal (Salamone et al., 2002, 2005, 2007). In fact, accumbens DA depletions alter effort-related choice (Salamone et al., 1991, 2007) and disrupt operant responding substantially in schedules with high effort demands (i.e., number of lever presses needed to receive reinforcement, progressive ratio test) (Aberman and Salamone, 1999; Salamone et al., 2001). In line with these results, previous studies using food pellets as a reward (non-psychostimulant rewards) reported that stimulation of accumbens $A_{2A}R$ disrupted the performance of an instrumental task with high work demands (using progressive ration schedule test) but had little effect on a task with a lower work requirement (Mingote et al., 2008). In addition to this, the same authors reported that the activation of accumbens $A_{2A}Rs$ by local injections of CGS 21680 increased extracellular GABA levels in the ventral pallidum (the indirect pathway of the ventrostriatal reward circuit. See sections 2.2.2.1 and 2.2.2.2). Similarly, specific D_2R upregulation in the indirect pathway (NAc projection to the ventral pallidum) promotes motivation for food by weakening the canonical output to the ventral pallidum (Gallo et al., 2018). Given that $A_{2A}Rs$ co-express with D_2R in the ventrostriatal indirect pathway and its antagonist interaction, $A_{2A}Rs$ can be proposed as a promising therapeutic target for the motivational symptoms, including those observed in eating disorders for palatable food, such as binge-eating disorder.

4 Complex multifactorial nature of the binge-eating disorder: gene and environment interaction

Binge-eating disorder implicates feeding beyond homeostatic needs in a short period, together with a sense of loss of control. Such mental disorder is typically chronic, episodic, and often associated with psychiatric comorbidity (such as bipolar disorders, depressive disorders, anxiety disorders, and, to a lesser degree, substance use disorders) and medical sequelae, resulting in significant personal, family, and social costs (see section 1.1.1). Therefore, this aberrant feeding behaviour integrates a complex multifactorial nature, including different brain areas and neuronal pathways and multiple intracellular pathways combined with genetic and multiple environmental factors that interact between them. These genetic and environmental factors influence brain development and function, altering behaviour and determining a person's vulnerability or resilience to developing the condition. Intrinsic (sex, age, personality traits, genetics, comorbidity with other psychopathological illnesses, among others) and extrinsic (socioeconomic position, unpleasant life experiences and anxiogenic environments, among others) factors contribute to this vulnerability. The proportional importance of these elements fluctuates throughout one's life and at various stages of the disease. This multifactorial nature explains why only a percentage of people develop binge-eating disorder, regardless of the genetic predisposition or the easy access to highly palatable food in our society.

4.1 Genetic mechanisms in the development of the binge-eating disorder

Disordered eating habits and dysfunctional eating attitudes, which are linked to eating disorders, are also influenced by genetic factors. Similar to anorexia nervosa and bulimia nervosa, binge-eating disorder is also found to aggregate within families (Fowler and Bulik, 1997) and in a manner independent of obesity (Hudson et al., 2006). The heritability estimates for binge-eating disorder range from 41% to 57% (Javaras et al., 2008; Mitchell, 2016). In addition, according to a large population-based twin study, individual differences in the binge-eating disorder can be explained by additive genetic factors (Munn et al., 2010). In fact, shared environmental factors do significantly affect disordered eating in adoptive siblings, and the heritability of disordered eating is high for twins reared apart (Klump et al., 2009), further highlighting the importance of genetic factors.

Different molecular genetic approaches have been applied to study eating disorders: linkage studies, candidate-gene association studies, genome-wide association study (GwAS) and study of rare variants. Given the documented serotonergic abnormalities in eating disorders using brain imaging studies, (Kaye, Bailer, et al., 2005; Kaye, Frank, et al., 2005), serotonergic genes and their involvement in eating disorders have been widely studied. Notably, overexpression of the long-allele of 5-HTTLPR was correlated with the binge-eating disorder compared to normal-weight controls (77 BED patients vs 66 normal-weight) (Monteleone et al., 2006). Similarly, dopaminergic genes have been broadly studied in the context of eating disorders, given their

essential involvement in a large variety of brain functions, including feeding behaviour, motor activity, and reward. The A1 allele of the dopamine D_2R gene (Taq1A) is the best characterized polymorphism. This polymorphism is located more than 10 kilobase pairs downstream from the coding region of the DRD_2 gene or in the coding gene for the neighboured ANKK1 gene (Neville, Johnstone and Walton, 2004). Individuals with the A1 allele of the Taq1A polymorphism (rs1800497) have been linked to the idea of "reward deficiency syndrome", consisting of a hypodopaminergic state caused by a faulty D_2R and lower D_2R density than other people. As a result of the lack of D_2R, people are at a higher risk of engaging in various addictive, impulsive, and compulsive behaviours. Following that, the Taq1A A1 allele has been directly linked to obesity and substance use disorders (Blum et al., 1996, 2000), and further studies in eating disorders revealed that D_2R and neighbouring ANKK1 SNPs were also implicated in binge eating (Davis et al., 2008, 2009, 2012). However, GwAS and sequencing studies have not been conducted in binge-eating disorder.

Recent research has begun to highlight the necessity of considering **epistatic interactions** among polymorphic loci when addressing the simultaneous participation of numerous genes in the regulation of pathways. Epistasis is a term used to describe a group of complicated interactions between genetic loci (Phillips, 2008). Nikolova et al. were the first to apply a physiologically based "multilocus genetic profile score", a composite genetic index that reflects the cumulative influence of numerous polymorphic loci with established functions on a given signalling pathway (Nikolova et al., 2011). As a result of the simultaneous assessment of many functional loci, polymorphisms with

non-significant independent effects can be included, which merely account for significant proportions of variability. They created a multilocus genetic profile that can be used to explain individual differences in reward-related ventral striatum variability by combining the impact of functional polymorphisms on DA signalling (comprising DAT, DRD_2, DRD_4, and COMPT genes), which have been individually linked to variation in striatal DA signalling. They discovered that the multilocus genetic profile score accounted for a more significant variance than the single locus genetic profile score. Studies employing the same multilocus genetic profile technique, which included six functional markers on four DA genes, reported a similar outcome in binge eating disorder. For example, binge-eating disorder patients had a considerably higher multilocus genetic profile score than obese non-binge eating disorder controls, indicating that binge-eating disorder patients have stronger DA signalling in the striatum and are more susceptible to reward (Davis, 2015).

Therefore, **binge-eating disorder is a multifactorial disease** with a polygenetic component (Volkow and Muenke, 2012). Because of that, it makes it more challenging to determine a person's risk of inheriting or passing this disorder. However, a multifactorial threshold model has been postulated (Reich, Cloninger and Guze, 1975). In this model, a number of different genes, and a number of environmental variables, are supposed to operate as risk and protective factors for the development of the disease and are grouped together as "liability." **Individual liabilities** in a population create a continuous variable that is regularly distributed throughout the population. If the combined impact of genetic and environmental variables pushes a person's liability over a certain threshold level, then the individual is affected (**Figure 21**).

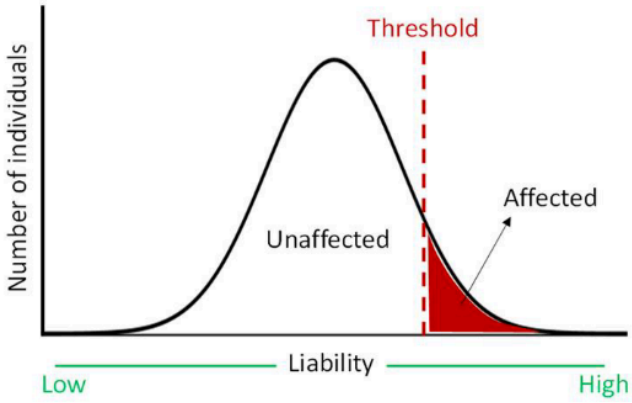


Figure 21. Multifactorial threshold model. The liability distribution for a multifactorial disease. An individual must exceed a threshold on this distribution to be affected with the disease

4.2 Epigenetic mechanisms in the development of the binge-eating disorder

Epigenetic pathways are promising prospects for research into psychiatric diseases induced by interactions between hereditary and environmental variables, such as histones modifications, DNA methylations and Non-coding RNA (microRNA). The term epigenetics refers to a set of biochemical processes that allow for changes in gene expression during an organism's life cycle without requiring a change in DNA sequence (Jaenisch and Bird, 2003). Consequently, epigenetic mechanisms can be seen as the vehicle through which the environment interacts with a person's genome to determine all aspects of health and disease function (**Figure 22**) (Nestler, 2014). Biologically, epigenetic mechanisms can be categorised into DNA modifications, histone modifications, and non-coding RNA.

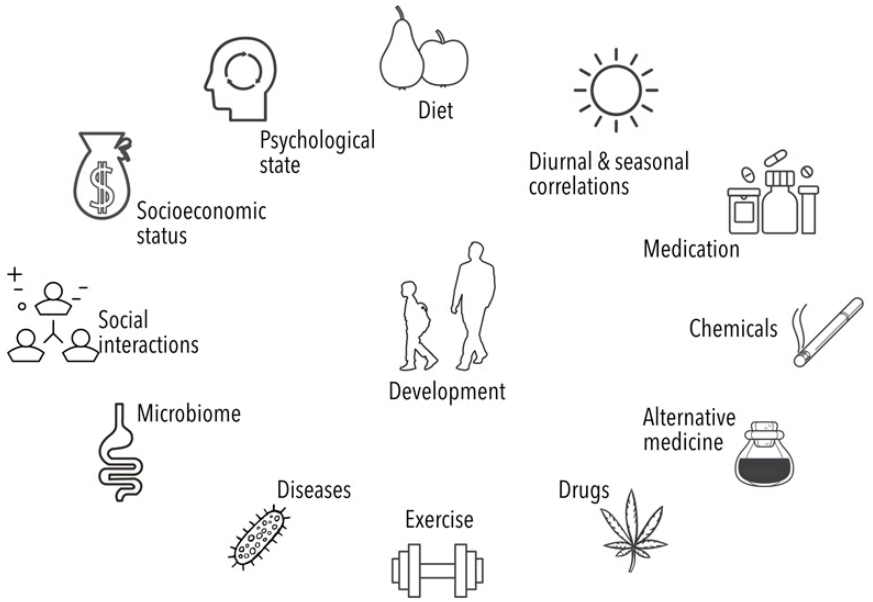


Figure 22. Environmental factors that influence epigenetic profiles. The assessment of these factors should be included in the design of a study investigating epigenetic profiles. Addapted from (Hübel, Sarah J Marzi, et al., 2019)

DNA methylation has been associated with a variety of psychiatric illnesses, including schizophrenia (Aberg et al., 2014; Hannon et al., 2016), Alzheimer's disease (de Jager et al., 2014; Lunnon et al., 2014), and autism spectrum disorder (Wong et al., 2014; Zhu et al., 2014). The oxidised derivatives of DNA methylation are also DNA alterations, with DNA hydroxymethylation gaining growing interest in neuropsychiatric disorder due to its abundance in the human brain (Kriaucionis and Heintz, 2009; Globisch et al., 2010). While epigenetic states were previously thought to occur independently of DNA sequence, current research has found evidence of extensive effects of genetic polymorphisms on epigenetic states. Methylation quantitative trait loci (mQTLs) are being studied with great interest. These are single nucleotide polymorphisms (SNPs) that

affect the methylation state of a CpG site, usually proximal to the SNP (Gibbs et al., 2010; McClay et al., 2015). Several characteristics of eating disorders support an investigation into the potential contribution of epigenetic factors, including sex differences (Steinhausen and Jensen, 2015), periods of increased risk of onset (Volpe et al., 2016) and reported discordance between monozygotic twins (Thornton et al., 2017; Kesselmeier et al., 2018). For example, eating disorders are associated with early life stress (Caslini et al., 2016) and emerging evidence links early life stress with epigenetic profiles. One methylome study of candidate genes in the binge-eating disorder has been reported (Jia et al., 2017; Veldic et al., 2017). The study primarily investigated promoter methylation of SLC1A2, a gene involved in glutamate clearance, in bipolar disorder. The authors found decreased DNA methylation in bipolar disorder patients who also suffered from binge-eating disorder, compared to those who were only affected by bipolar disorder. However, their sample of patients reporting binge-eating behaviour seemed to comprise bulimia nervosa and binge-eating disorder cases, rendering the interpretation of the results ambiguous (Jia et al., 2017; Veldic et al., 2017).

Although empirical evidence confirms the association between early life factors and epigenetic profiles in humans (Szyf and Bick, 2013), the conclusions are limited due to the low availability of brain tissue and the scarcity of extensive longitudinal studies that collect information on early traumatic experiences and biological samples enabling epigenetic analysis (Provençal and Binder, 2015). Furthermore, the most extensive study of early-life adversity and DNA methylation in blood published before 2019 identified no significant differential methylation (Marzi et al., 2018). These characteristics suggest that the interaction of genetic risk

factors and environmental stressors can contribute to the onset of eating disorders and make them an excellent target for examining epigenetic effects on appetite regulation and eating behaviour. Although epigenetic research in eating disorders, especially in binge-eating disorder, is still in its early stages, preliminary findings from pilot studies stimulate further larger-scale research.

4.3 Transcriptomic studies and limitations

As mentioned above, epigenetic profiling studies in humans are still limited. This is due to the low availability of brain tissue and the scarcity of extensive longitudinal studies collecting information on early traumatic experiences and biological samples, which allow reliable epigenetic analysis. However, a proper combination of genetic studies and behavioural models allows more translational approaches that integrate genetic and environmental factors. In the case of eating disorders, animal models' preclinical studies are an essential tool to investigate possible target genes implicated in regulating neuronal pathways associated with specific conditions.

Transcriptomic techniques have been developed to study and analyse gene expression to understand the relationship between the genome and the functioning of cells. This analysis describes a cell's potential functions and its response to extracellular signals. The **significant directions of transcriptomics** studies are **1)** to characterise different states of cells (i.e. development stages), **2)** tissues or cell cycle phases by expression patterns; **3)** explore the molecular mechanisms underlying a phenotype; **4)** identify biomarkers differently expressed between the diseased state and healthy state; **5)** distinguish disease stages or subtypes

(e.g. cancer stages); **6)** establish the causative relationship between genetic variants and gene expression patterns to illuminate the aetiology of diseases (Schadt et al., 2005).

Transcriptomic is the study of the "transcriptome", a term credited to Charles Auffray for the first time to refer to a complete set of transcripts (Piétu et al., 1999). The term transcriptome today refers to the whole collection of all ribonucleic acid (RNA) molecules expressed in a particular entity, such as a cell, tissue, or organism (Morozova, Hirst and Marra, 2009). Modern transcriptomics uses high-throughput technologies to examine the expression of many transcripts in a variety of physiological and pathological circumstances, rapidly expanding our knowledge of the link between the transcriptome and phenotype in a broad spectrum of living organisms.

The gene expression analysis *in vivo* confronts the problem that all tissues contain various cell types with unique functions. The brain is a particularly difficult tissue to quantify in terms of gene expression and translation. For example, around 75 million neurons, 23 million glial cells, 7 million endothelium cells, and several million additional cell types make up a typical mouse brain (Williams, 2000). Explicitly focused on neurons, they are exceedingly diverse in their expression of multiple neurotransmitters, neuropeptides, and receptors, and they are generally physically mixed, making molecular study difficult. Given this complexity, different approaches have been developed to isolate specific cell types to investigate their role in particular environments or diseases. However, each method has advantages and disadvantages.

One of them is **cell sorting** based on the promoter-specific expression of fluorescent marker proteins. This **sorting technique** to isolate a specific

cell type for analysis can be successful, but additional manipulations are both time-consuming and have been shown to alter transcription (Haimon et al., 2018). This isolation cell strategy allows to identify of marked cells in a complex organ such as the brain using manual dissection, enzymatic dissociation combined with fluorescence-activated cell sorting (FACS), or laser capture microdissection (LCM) (Buchstaller et al., 2004; Arlotta et al., 2005; Yao et al., 2005; Lobo et al., 2006; Rossner et al., 2006; Sugino et al., 2006; Hempel, Sugino and Nelson, 2007). Although the sorting techniques (using FACS and LCM) provide a mechanism for isolating specific cell types from a complex mixture, they present several obstacles to successfully isolating cell-type-specific transcripts. With manual dissection or enzymatic dissociation combined with FACS, the three-dimensional relationship of multiple cell types present in a tissue must be wholly disrupted, often via enzymatic dissociation, and this may lead to changes in gene and protein expression (Yao et al., 2005; Rossner et al., 2006; Sugino et al., 2006). In the case of LCM, cells are removed from their *in situ* environment, but the tissue must be sectioned, processed, and dehydrated. Furthermore, only the cell bodies are captured in cells with very complex anatomy, such as astrocytes and neurons in the brain, leaving dendritic and axonal mRNAs behind. Bearing this in mind, an alternative isolation method is the **ribosome-tagging approach (RiboTag)** (Sanz et al., 2009).

4.4 Cre:RiboTag

To address the ongoing challenge in defining transcriptional and translational changes in specific cell populations in complex tissues, Sanz *et al.*, (2009) developed a method to tag ribosomes in specific cell types

using Cre-loxP-dependent recombination (Cre:RiboTag). This approach consists of a line of transgenic mice (RiboTag) carrying a ribosomal protein allele (Rpl22) with a floxed wild-type C-terminal exon followed by an identical C- terminal exon that has three copies of the hemagglutinin (HA) epitope inserted before the stop codon. This epitope-tagged ribosomal protein allele ($Rpl22^{HA}$) can be activated in specific cell types by mating to a Cre recombinase-expressing mouse, incorporating the $Rpl22^{HA}$ into actively translating polyribosomes. The epitope-tagged polyribosomes can be purified from the target cell population via immunoprecipitation with a monoclonal antibody against HA and subsequently analysed using standard genomic profiling technologies such as quantitative RT-PCR (qRT-PCR), microarray and RNA-sequencing (**Figure 23**).

Exist similar approaches based on a series of bacTRAP transgenic mice to drive expression of an EGFP-tagged ribosomal protein in specific neuronal cell types, allowing the use of immunoprecipitation techniques to isolate polysome-bound mRNA (Doyle et al., 2008; Heiman et al., 2008). However, the **Cre:Ribotag** technique has **two principal advantages** over this technique. While the bacTRAP technique depends on expression levels from cell-type-specific promoters that are quite variable in transcriptional activity and generally much lower than competing ribosomal protein promoters, the modified endogenous Rpl22 is high and proportional to the ribosomal content of the cell. A second major advantage is that the RiboTag methodology takes advantage of the full range of Cre recombinase-expressing mouse lines that have already been created, including knock-in Cre-expressing mice that may more accurately replicate the endogenous cell-type specificity of particular genes. Moreover, by using the RiboTag technology, it is also possible to avoid

concerns about the random insertion of the BAC construct into the mouse genome and the observation that BACs often contain additional intact genes that may produce phenotypes and complicate interpretation of the data (Heiman et al., 2008).

Specifically, in this study, we took advantage of this technology to analyse the transcriptomic profile of D2 (+) neurons using D2-Cre:Ribotag transgenic animals, already validated by (Puighermanal et al., 2020) (**Figure 23**).

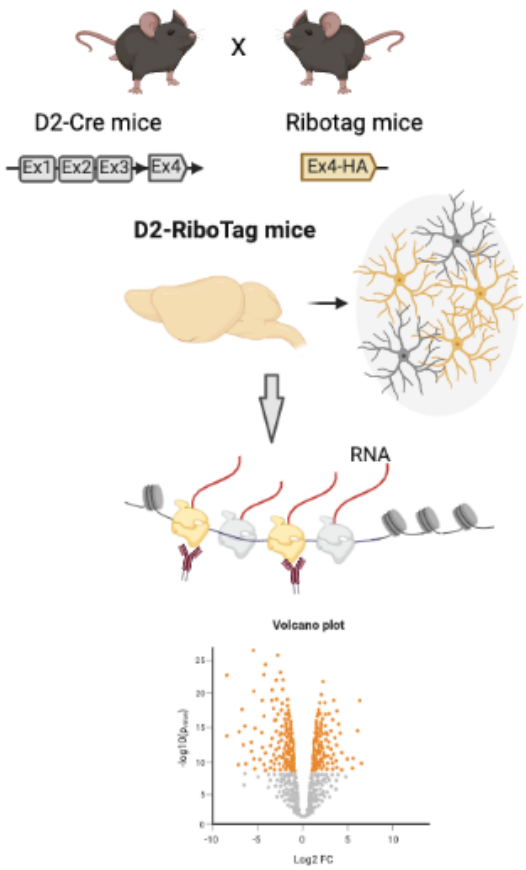


Figure 23. Schematic representation that outlines the RiboTag methodology. The RiboTag mouse is crossed to D2-Cre re recombinase-expressing mouse line,

which results in the deletion of wild-type exon 4 and replacement with the HA-tagged exon 4 only in cells that express D2-Cre recombinase.

5 Binge eating mouse models

Binge-eating behaviour has been studied using various animal models, including the use of cyclic periods of food deprivation (fasting) followed by feeding to encourage animals to compulsive binge eat after palatable food presentation or the use of stressors to trigger binge-eating episodes (Avena and Bocarsly, 2012). The use of stressors allows reproducing the environmental conditions responsible for provoking in some cases the compulsive overeating reported in patients suffering from binge-eating disorder. However, these models have some disadvantages. For example, overnight food deprivation causes hyperphagia, but at the same time, causes increased locomotor activity and significantly elevates corticosterone levels. Similarly occurs in the case of a combination of caloric restriction and stressors like footshock that produces binge-like eating in rats (Hagan et al., 2002). Furthermore, some of these models typically do not produce stable binge-like eating patterns until 2-8 weeks after initiation of the protocol, requiring a significant time investment from the researcher (Hagan and Moss, 1997; Dimitriou, Rice and Corwin, 2000; Davis et al., 2008). Therefore, the reliable induction of binge-like eating behaviour in rodents is dependent on numerous factors, including genetic background, postnatal rearing environment and schedule of access conditions to palatable foods (Hagan et al., 2003; Wojnicki, Johnson and Corwin, 2008; Cifani, Polidori, et al., 2009; Cifani, Zanoncelli, et al., 2009). Another alternative behavioural approach to reproduce binge-like eating in mice was based on successive cycles of food restriction

with repeated exposure to forced swim-induced (Consoli et al., 2009). However, this behaviour could not be maintained beyond three cycles once initiated. Thus, developing a mouse model of binge-like eating behaviour has proven to be more complex because minor stresses can significantly inhibit food intake in this species (Teegarden and Bale, 2008).

Consequently, some laboratories developed a rapid and relatively simple model of binge-eating behaviour in mice that do not require food deprivation nor the application of exogenous stressors (Cottone et al., 2009; Czyzyk, Sahr and Statnick, 2010). This protocol consists of the cyclical reproduction of binge eating episodes. Mice are exposed to intermittent access to palatable food only for 24h/48h (free-choice access to palatable food period) followed by *ad libitum* access to standard food during the following 6/5 days. Then, the binge-eating behaviour (binge eating episodes) is studied during the free-choice period by assessing food intake at 2.5 hours, 24 hours, and 48 hours. By using this behavioural approach is possible to investigate the intrinsic features of the palatable food, as well as to determine the individual traits in a heterogeneous population that may cause binge-like eating behaviour without using exogenous conditions (fasting or stressors) that may increase the percentage of individual vulnerability to compulsive overeating.

As mentioned above (see section 1.1.1.1), binge-eating episodes are characterised by an abnormality in the amount of food consumed than by craving for a specific nutrient since the type of food consumed during binges varies across individuals (American Psychiatric Association, 2013). However, animal models based on access to palatable food such as **high energy diet** (HED) (73% more fat and 43% more sucrose than standard food) (Czyzyk, Sahr and Statnick, 2010) or **cafeteria diet** (CD) (composed

of an equivalent mixed of four popular brand chocolate bars highly consumed in humans) (Gutiérrez-Martos et al., 2018) allow mimicking the binge eating episodes observed in humans. Mice following the previously described protocol consumed an average of 1.2 g of HED during the initial 2.5 h, equivalent to one-third of the total daily caloric intake of non-bingeing mice. Moreover, all mice in the intermittent group exhibited binge-like eating and preferred the HED over the control group (standard chow) (Czyzyk, Sahr and Statnick, 2010). In addition to this, Czyzyk et al. did not report evidence of entrainment of food intake behaviour in the intermittent group since levels of HED intake were consistent each week. In addition, this model has predictive validity since some pharmacological agents with clinical efficacy in binge eating disorder reduce binge eating in this model. However, this mouse model does not represent other aspects of human illness, such as increases in serum corticosterone levels or anxiety and depressive-like behaviours (Novelle and Diéguez, 2018). Despite that, this behavioural model is a reliable and consistent protocol that allows preclinical studies to investigate binge eaters' neurobiology and genetic profiles compared to different groups (such as obese non-binge eaters and standard weight). Moreover, this protocol can be combined with well-established protocols to investigate the underlying neurobiological mechanisms of binge-eating disorder compared to other overeating disturbance like food addiction.

6 Therapeutics approaches for treating eating disorders

Binge-eating disorder is not a metabolic disorder and is not always associated with obesity. This disorder can cause significant emotional and

physical distress, important medical issues, and an increased mortality rate (NEDA, 2016). Because of that, the first-line treatment for the binge-eating disorder in adults is individual **psychological therapy**.

Like other eating disorders, binge-eating disorder requires a big-picture treatment plan tailored to meet the individual needs to help the patients gain control over their eating behaviour. Most often, it involves a combination of strategies.

6.1 Non-pharmacological treatments: Psychotherapy

6.1.1 Behavioural therapies

This type of individual counselling focuses on changing thinking (cognitive therapy) and behaviour (behavioural therapy). It offers ways to cultivate healthy attitudes toward food and weight and methods to alter reactions to challenging situations. This non-pharmacological treatment is based on three main strategies: **cognitive behaviour therapy** (CBT), **interpersonal psychotherapy** (IPT) and **dialectical behaviour therapy** (DBT).

CBT and IPT are special therapies for the binge-eating disorder that directly target the eating disorder, but they are different. CBT focuses on helping the patient cope better with issues that can trigger binge-eating episodes, such as negative feelings about the body or a depressed mood and may also give a better sense of control over behaviour and help regulate eating patterns. On the other hand, IPT focuses on relationships with other people. The goal is to improve interpersonal skills (relationships with others, including family, friends and co-workers). This therapy aims to help reduce binge eating triggered by problematic relationships and unhealthy communication skills. These interventions have been shown in

well-controlled studies to help eliminate binge eating and lower related psychopathology in the short and long term, but they do not generate clinically significant weight loss on average (Wilfley et al., 2002; Wilson, Grilo and Vitousek, 2007).

Although binge-eating disorder and obesity are independent, they are usually associated. For that reason, **behavioural weight-loss programs** used to be included in the treatment. However, many people with binge-eating disorder have a history of failed attempts to lose weight on their own. Because of that, weight-loss programs typically are not recommended until the binge-eating disorder is treated since dieting may trigger more binge-eating episodes, making weight loss less successful.

6.1.2 Neurostimulation therapies

The circuit-based therapeutics could be invasive as deep brain stimulation or non-invasive, such as repetitive transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation (tDCS).

Deep brain stimulation (DBS)

DSB is a non-lesional invasive neurosurgery method involving the surgical implantation of current-passing electrodes to stimulate discrete brain areas electively. It has been used as a potential treatment for several circuit-based neuropsychiatric conditions, including obsessive-compulsive disorder (Alonso et al., 2015) and major depression (Cleary et al., 2015). It is well established as a safe and effective treatment for Parkinson's disease (Honey et al., 2017). On the other hand, deep brain stimulation is a nonspecific stimulation that lacks the selectivity of preclinical optogenetic techniques (Cooper, Robison and Mazei-Robison, 2017). In the field of addiction, a combination of deep brain stimulation and pharmaceutical

adjuvant that helps to remove opposing effects from general stimulation has been presented as a solution to this problem. This technique was also sufficient in preclinical models to counteract cocaine-induced alterations in synaptic plasticity and cocaine locomotor sensitisation in cocaine-exposed mice (Creed, Pascoli and Lüscher, 2015).

DBS has also been employed in obesity research, both preclinical and clinical. Women lost weight and had a lower BMI after activation of specific hypothalamic areas (homeostatic centre) and NAc (reward system) (Harat et al., 2016). These findings suggested that DBS could be used to treat obesity, food addiction, and other eating problems, such as binge-eating disorder. However, further research is needed to understand better the potential adverse effects and effectiveness of DBS in this field.

Repetitive transcranial magnetic stimulation (rTMS) and transcranial direct current stimulation (tTMS)

rTMS and tDCS are non-invasive stimulation techniques that do not require surgery. rTMS delivers weak electrical current to brain regions through electrodes placed on the scalp to either depolarise (anodal tDCS) or hyperpolarise (cathodal tDCS) resident neurons, while tDCS delivers weak electrical current to brain regions through electrodes placed on the scalp to either depolarise (anodal tDCS) or activate (high frequency, >5 Hz rTMS) target neurons. To present, the majority of studies have used high-frequency rTMS and tDCS to improve neuronal excitability and cortical activity in the dlPFC, hence increasing executive function.

Because of its connection to the limbic system, the dlPFC is known to exercise top-down control (Volkow et al., 2013). Obese people and drug addicts have lower dlPFC activity, which is linked to obsessive behaviours

(Lindgren et al., 2018). In patients with cocaine use disorder, recent trials using high-frequency rTMS of the dlPFC indicated reduced cocaine usage and cravings (Terraneo et al., 2016). After five weeks of high-frequency dlPFC rTMS, a cohort of obese participants reported reduced food cravings and weight loss (Ferrulli et al., 2019). Similarly, other studies using tDCS to stimulate the dlPFC in obese patients found a reduction in food cravings (Lee, Elias and Lozano, 2018).

The production of long-term neuroplastic alterations affecting cortical excitability could explain the efficacy of high-frequency rTMS of the dlPFC in treating addiction-like behaviour towards drugs or food. Due to the release of a wide variety of neurotransmitters, long-term plasticity is formed at dlPFC, the primary activation site, and may affect on subcortical areas, the secondary activation sites. Indeed, rTMS of the dlPFC at high frequencies causes a prolonged increase in DA levels in the human ventral striatal complex (Diana et al., 2017). Given its safety-feasibility profile and lack of significant side effects, these findings imply that rTMS and tDCS could play a role in treating obesity, food addiction, and addiction. However, more research is needed to determine stimulation parameters, treatment frequency, and the long-term sustainability of any positive effects.

6.2 Pharmacological treatments

Several therapeutic drugs have been used to control body weight and binge-eating episodes. **Lisdexamfetamine dimesylate** (**Vyvanse**), a drug for attention-deficit hyperactivity disorder (ADHD), was the first FDA-approved medication to treat moderate to severe binge-eating disorder in adults. Lisdexamfetamine is an inactive prodrug that is converted in the

body to dextroamphetamine, a pharmacologically active compound which is responsible for drug's activity. The optical isomers of amphetamine, (i.e., dextroamphetamine and levoamphetamine) are TAAR1 agonists and vesicular monoamine transporter 2 inhibitors that can enter into monoamine neurons; this allows them to release monoamine neurotransmitters (dopamine, norepinephrine and serotonin, among others) from their storage sites in the presynaptic neuron, as well as prevent the reuptake of these neurotransmitters from the synaptic cleft (Eiden and Weihe, 2011). Vyvanse has been studied in three trials and was associated with reductions in binge episodes per week, decreased eating-related obsessions and compulsions, and reductions in weight (Slomski, 2015). However, this treatment is a stimulant that can be habit-forming and abused and causes common side effects, such as dry mouth and insomnia, and more severe side effects.

Other medications are anticonvulsant medications, particularly **topiramate** (available as Trokendi XR, Qudexy XR, and Topamax). This drug has also been studied, and there is limited evidence suggesting its usefulness. However, a clinical study reported that prolonged administration of topiramate induces a marked reduction in the frequency of binge episodes with significant weight loss (Milano et al., 2013). Topiramate is a medication with GABAergic activity and antagonizes AMPA/kainate glutamate receptors agonist activity, decreasing appetite and weight. The possible side effects include dizziness, nervousness, sleepiness and trouble concentrating.

Additional anti-obesity medications have been licensed based on evidence suggesting the neurological mechanisms underlying food addiction, eating disorders, obesity, and drug addiction are mostly similar.

As a result, novel treatments for obesity and food addiction have been proposed based on medications that are effective in treating drug addiction (Lindgren et al., 2018), but its usefulness in binge eating control is not tested. **Naltrexone** is an opioid antagonist that works primarily on the mu-opioid receptor and is used to treat alcohol and opioid addiction. Naltrexone use has been shown to reduce food intake and decrease subjective like of foods, particularly highly palatable food, in normal-weight individuals. Furthermore, when normal subjects see and taste chocolate, naltrexone lowers reward activation (Lee and Fujioka, 2009). Another medication that may help reduce binge-eating disorder symptoms are antidepressants such as **bupropion.** This medication is a norepinephrine-dopamine reuptake inhibitor leading to an increased DA activity, currently approved for smoking cessation. Combining Naltrxone and Bupropion sold under the brand name, **Contrave** has been proposed to be a new anti-obesity treatment. Contrave administration reduces body weight (5% or more) after 56 weeks of treatment in 48% of obese participants (Greenway et al., 2010). Moreover, Contrave blunted hypothalamic activation to food cues and enhanced activation of brain regions involved in inhibitory control, internal awareness and memory (G. J. Wang et al., 2014).

The CB_1R antagonist medication, **rimonabant**, is worthy of mentioning. This drug treatment is an important brain reward modulator in the endocannabinoid system, which controls appetite and consumption of food approved in Europe and more than 30 countries worldwide in 2006 to treat obesity. Although effective in inducing weight loss, this drug was withdrawn from clinical use just two years later due to psychiatric side effects, including anxiety, depression and suicidal ideas. Afterwards, new

neutral CB_1R antagonists and/or peripherally restricted CB_1R antagonists unable to cross the blood-brain barrier have been developed. Alternatively, pharmacological modulators of endocannabinoids synthesis, rather than CB_1R blockade, could provide a more physiological approach to treat obesity and binge-eating episodes.

Objectives

General objective

The overall purpose of this Doctoral Thesis was to characterise the specific transcriptional changes in NAc D2(+) neurons and neuronal pathways in the brain's reward system that could unravel the neuropathological mechanisms involved in the compulsive-like behaviour over food intake described in the binge-eating disorder, as well as to develop a translational mouse model of BED based on psychiatric diagnostic manual criteria.

Objective 1.

To investigate the influence of dietary condition on gene expression of D2(+) neurons in the nucleus accumbens to distinguish the particular functioning of this subtype of neurons during uncontrolled food intake.

Study 1*: Endogenous depletion of 2-AG levels in the nucleus accumbens reduce palatable food intake in binge-like eating mice*

Objective 2.

To study the role of adenosine A_{2A}Rs in the indirect pathway of the nucleus accumbens in the regulation of binge-like eating episodes.

Study 2: *Adenosine A_{2A} receptor overexpression into the nucleus accumbens to ventral pallidum pathway decreases motivation for chocolate in binge-like eating mice*

Objective 3.

To develop a rodent model and analysis method using the PheComp cages to discriminate vulnerable traits for the development of binge-eating disorder in a heterogeneous population of mice based on the diagnostic criteria described in the diagnostic and statistical manual of mental disorders (DSM-5, 2013).

Study 3: *Behavioural characterization of a mouse model of binge-like eating behaviour in PheComp cages.*

Material and methods

Animals

Male mice from 8 to 12 weeks old at the beginning of the experiment were used. Animals were singled housed and maintained with food and water *ad libitum* in a controlled temperature (21 ± 1°C) and humidity room (55 ± 10%) with a 12:12-hour reversed light/dark cycle (off at 8 a.m. and on at 8 p.m.). Mice were habituated to the experimental room and handled for one week before starting the experiments, and all the tests were performed during the dark phase of a reverse light cycle. Firstly, we used *D2-Cre:RiboTag* (C57BL/6J background; Puighermanal et al., 2020) and their wild type (WT) littermates C57BL/6J. We used WT C57BL/6J mice purchased from Charles River (France) for the following two studies. All experimental protocols were performed in accordance with the guidelines of the European Communities Council Directive 2010/63/EU and approved by the local ethical committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA PRBB, agreement N°9687 or by the French Agriculture and Forestry Ministry (A34-172-13)). Maximal efforts were made to reduce the suffering and the number of mice used.

Drugs

For the surgical procedure, ketamine hydrochloride (Imalgène; Merial Laboratorios S.A.) and medetomidine hydrochloride (Domtor; Esteve, Spain) were mixed and diluted in sterile 0.9% physiological saline and administered intraperitoneally (i.p, 75 mg/kg and 1 mg/kg of body weight respectively) to anaesthetize the mice. After surgery, anaesthesia was reversed by a subcutaneous (s.c.) injection of atipamezole hydrochloride (Revertor; Virbac, Spain; 2.5 mg/kg of body weight) diluted in sterile 0.9% physiological saline. In addition, mice received after surgery a preventive treatment with an i.p. injection of the antibiotic gentamicin (Genta-

Gobens; Laboratorios Normon, Spain; 1 mg/kg of body weight) and a s.c. injection of the anti-inflammatory meloxicam (Metacam; Boehringer Ingelheim, Rhein; 2 mg/kg of body weight) both diluted in sterile 0.9% physiological saline.

Different compounds were administered bilaterally into the nucleus accumbens (NAc) level for the pharmacological validation of the functional involvement of each candidate gene. A selective inhibitor of 2-AG biosynthesis via DAGLα (diacylglycerol lipase α), 2-[(fluoro-methyl phosphinyl)oxy]-1-[(1-methylethoxy)methyl]ethyl ester, 9Z-octadecenoic acid (O-7460), (Cayman Chemical, Michigan, USA) was dissolved in 5% DMSO and physiological saline (0.9% NaCl) and administered at two doses, 1ng/side and 10ng/side. The selective agonist of CCK receptor, CCK Octapeptide, sulphated (CCK8-S) (Tocris, Bio-Techne R&D Systems, S.LU) was dissolved in physiological saline (0.9% NaCl) and administered at two doses, 1ng/side and 2ng/side. Selective adenosine 2 receptor agonist 2-p-(2-carboxyethyl) phenethylamine-5'-N-ethylcarboxamidoadenosine (CGS-21680) (Sigma-Aldrich, Sant Louis, Missouri, USA) was dissolved in physiological saline (0.9% NaCl), sonicated for 8-10 min and administered at two doses, 1ng/side and 2ng/side. Immediately after administration, animals were evaluated in the different behavioural paradigms.

Surgeries

General surgeries procedures

Mice were anaesthetized as reported in the "Drugs" section in material and methods and placed into a stereotaxic apparatus for receiving both types of surgeries: fixed cannula implantation and virus vector microinjection. All the intracranial injections, drug and viral

microinjection, were made through a bilateral injection cannula (33-gauge internal cannula, Plastics One, UK) connected to a polyethylene tubing (PE-20, Plastics One, UK) attached to a 10 µl microsyringe (Model 1701 N SYR, cemented NDL, 26 ga, 2 in, point style 3, Hamilton company, NV). The displacement of an air bubble inside the length of the polyethylene tubing that connected the syringe to the injection needle was used to monitor the microinjections.

Fixed cannula implantations into the NAc for bilateral administrations

Anaesthetized WT C57BL/6J mice were placed into a stereotaxic apparatus to implant bilateral intracranial guide cannula (26-gauge external cannula, Plastics One, UK) 1mm above NAc according to Paxinos and Franklin mice's brain atlas (surgical coordinates: AP: 1.34 mm, L ± 1 mm, DV -3.6 mm). First, a skin incision was made over the skull to drill the holes over the target sites. Then, the bilateral cannula was inserted 1mm above NAc and secured in place with orthodontic acrylic (Dentalon® Plus, Kulzer) and an extra small screw. After recovering days, the intracranial injections of each drug through a bilateral injection cannula were made before the behavioural paradigm evaluations. Bilateral microinjections were delivered on a constant fixed rate of 0.25 µl/min to a final volume of 0.5 µl/side through a bilateral-injection cannula 1mm longer than the fixed guide cannula by using a microinfusion pump (Harvard Apparatus, Holliston, MA) for 2 min. The bilateral-injection cannula was kept inside the fixed guide cannula for an extra 1-minute period to avoid reflow of the injected volume. Animals were gently immobilized (previous habituation to the procedure) during the administration to avoid the cannula's damage or the subjects' injury. The fixed guide cannulas were protected with a

bilateral dummy-cannula (0.008inch, Plastic One, UK) 0.5mm longer than the guide cannula to avoid clogging that could hamper the fluid flow, and covered with a dust-cap (Plastic One, UK).

Histological validation was performed at the end of the experimental procedure to ensure the correct placement of the injection into the NAc. Mice were deeply anaesthetized by i.p. injection (0.2 ml/10 g of body weight) of a mixture of ketamine/medetomidine before administering methylene blue (M9140, Sigma Aldrich) through each injection-cannula. Subsequently, brains were extracted and immediately frozen using methyl butyrate (246093, Sigma Aldrich) cooled in dry iced. Coronal frozen sections (30 µm) of the NAc were obtained with a cryostat (CM3050, Leica Biosystem, Nussloch), mounted onto gelatinized glass slides, and stored at -20°C until use. Sections of NAc were observed under a Macro Zoom Fluorescence Microscope (MVX10, Olympus, Tokyo, Japan) to assess injection localization and histological features. Mice with incorrect placement in one or both hemispheres were excluded from the study.

Virus vector microinjection

The adeno-associated virus (AAV) intracranial injections volume was 0.4 µl per site in nucleus accumbens core (NAc core) and 0.2 µl per site in Ventral Pallidum (VP), injected at a constant rate of 0.1 µl/min (NAc core) and 0.05 µl/min (VP) by using a microinfusion pump (Harvard Apparatus, Holliston, MA) for 4 min. After infusion, the injection cannula was left in place for an additional period of 10 min to allow the fluid to diffuse and prevent reflux, and then it was slowly withdrawn for ten additional min. We used the following coordinates to target our injections according to Paxinos and

Franklin mice's brain atlas: (NAc core) AP + 1.34 mm, L ± 1 mm, DV − 4.6 mm; (VP) AP + 0.04 mm, L ± 1.5 mm, DV − 5 mm. The vectors used were: AAV-Adora2aR-mCherry (AAV8-hSyn-DIO-Adora2a-mCherry, 1,06E+13 gc/ml) and AAV-control-mCherry (AAV8-hSyn-DIO-mCherry, 1.19E + 13 gc/ml) from Viral Vector Production Unit of Universitat Autònoma de Barcelona, and AAV-retrograde-Cre-BFP (AAVrg pmSyn1- EBFP-Cre; 6×10^{12} vg/ml) from Addgene (viral prep # 51507-AAVrg). For the specific over-expression of $A_{2A}R$ in the projecting neurons from NAc to VP, two bilateral injections were performed in WT C57BL/6J mice, targeting the NAc core and the VP. Mice received an injection of 0.4 µl per site of the AAV-Adora2a-mCherry or AAV-control-mCherry into NAc core and an injection of 0.2 µl per site of the AAV-retrograde-Cre-EBFP into the VP. To detect the viral expression in all the experiments, we visualized each reporter listed below:

To detect AAV8-hSyn-DIO-Adora2a-mCherry and AAV8-hSyn-DIO-mCherry in WT mice, we directly visualized mCherry in the confocal microscope. mCherry is a bright red monomeric fluorescent protein visible in our experimental conditions without performing immunofluorescence.

To detect AAVrg-pmSyn1-Cre-EBFP targeting the VP, we performed immunofluorescence immunocytochemistry against Cre to detect the correct viral infection in the VP and visualized the labelled Cre in the confocal microscope (see section "immunofluorescent study").

Behavioural experiments.

Model of binge-like eating behaviour

We used a model of binge-like eating behaviour similar to previously published (Czyzyk, Sahr and Statnick, 2010). Mice were fed with standard food pellets (StdF) during the entire experimental period and were

exposed to a binge-like eating cycle (BC) once a week for a total of 6 weeks. Each BC consisted of *ad libitum* access to water and StdF (3.52 kcal/g) and high palatable pellets of cafeteria diet (CD) composed of an equitable mixed of four popular brand chocolate bars highly consumed by humans (MILKA®, SNICKERS®, BOUNTY® and MARS®, total 4.92 kcal/g) for 24 hours. Before beginning the experimental protocol, pre-exposure to CD for 48-h was performed to avoid neophobia to new flavours and food. Bodyweight and food intake (StdF and CD) were evaluated during each BC at different time points.

Self-administration behavioural model

Operant behaviour apparatus

Mouse operant chambers (Model ENV-307A-CT, Med Associates, Georgia, VT, USA) were used for operant responding maintained by chocolate-flavoured pellets. The operant chambers were equipped with two retractable levers, randomly selecting each chamber's active and inactive lever before starting the protocol. Pressing on the active lever resulted in a chocolate-flavoured pellet delivery paired with a stimulus-light (associated-cue) located above the active lever. Inversely, no consequences resulted after pressing on the inactive lever. A food dispenser equidistant between the two levers permitted the delivery of food pellets when required. The floor of the chambers was a grid floor that served to 43 deliver electric food shocks in the session of shock-test. The chambers were made of aluminium and acrylic and were housed in sound- and light-attenuated boxes equipped with fans to provide ventilation and white noise.

Food pellets

During the operant conditioning sessions, after active responding by lever pressing, animals received a 20 mg chocolate-flavoured pellet, a highly palatable isocaloric pellet (TestDiet, Richmond, IN, USA). These pellets had a similar caloric value (3.44 kcal/g: 20.6% protein, 12.7% fat, 66.7% carbohydrate) of standard maintenance diet provided to mice in their home cage (3.52 kcal/g: 17.5% protein, 7.5% fat, 75% carbohydrate) with some slight differences in their composition: addition of chocolate flavour (2% pure unsweetened cocoa) and modification in the sucrose content. Indeed, although the carbohydrate content was similar in the standard diet (75%) and highly palatable isocaloric pellets (66.7%), the proportion of sucrose content in standard diet food was 8.3% and 50.1% in highly palatable isocaloric pellets.

Protocol

The beginning of each self-administration session was signalled by turning on a house light placed on the chamber ceiling during the first 3 seconds (sec). The self-administration sessions were composed of two pellet periods (25 mins), separated by a pellet-free period (10 min). Pellets were delivered contingently after an active response paired with a stimulus light (cue light) during the pellet periods. A time-out period of 10 sec was established after each pellet delivery where the cue light was off, and no reinforcer was provided after responding on the active lever. Responses on the active lever and all the responses performed during the time-out period were recorded. During the pellet-free period, no pellet was delivered, and this period was signalled by the illumination of the entire self-administration chamber. In the operant conditioning sessions, mice

were under fixed-ratio 1 (FR1) (one lever-press resulted in one pellet delivery) for one week in the training phase, followed by an increased FR to 5 (FR5) (five lever-presses resulted in one pellet delivery) for the second part of the training phase and the rest of the sessions. As previously described (Martín-García et al., 2011), the criteria for the achievement of the operant responding were acquired when all of the following conditions were met: (1) mice maintained a stable responding with <20% deviation from the mean of the total number of reinforcers earned in three consecutive sessions (80% of stability); (2) at least 75% responding on the active lever; and (3) a minimum of 5 reinforcers per session. After each session, mice were returned to their home cages.

Different self-administration behavioural tests using operant behaviour apparatus were used to evaluate the vulnerability to food addiction-like criteria recently described (Mancino et al., 2015) and adapted from cocaine addiction-like in rats (Deroche-Gamonet, Belin and Piazza, 2004). These three criteria summarized the hallmarks of addiction based on DSM-5 and now included in the food addiction diagnosis through the YFAS 2.03.

Persistence to response: Non-reinforced active responses during the pellet-free period (10 min), when the box was illuminated and signalled pellet delivery unavailability, was measured as persistence of food-seeking behaviour. The last three consecutive days of the self-administration period were considered.

Motivation: The progressive ratio schedule of reinforcement was used to evaluate the motivation for the chocolate-flavoured pellets. The response

required to earn one single pellet escalated according to the following series: 1, 5, 12, 21, 33, 51, 75, 90, 120, 155, 180, 225, 260, 300, 350, 410, 465, 540, 630, 730, 850, 1000, 1200, 1500, 1800, 2100, 2400, 2700, 3000, 3400, 3800, 4200, 4600, 5000, and 5500. The maximal number of responses that the animal performs to obtain one pellet was the last event completed, referred to as the breaking point. The maximum duration of the progressive ratio session was 5 h or until mice did not respond on any lever within 1 h.

Compulsivity: Animals were exposed to conditional foot-shock test. In this shock session, mice were under an FR5 schedule of reinforcement during 50 min with two scheduled changes: at the fourth active lever-response mice received only an electric foot-shock (0.18 mA, 2 sec) without pellet delivery, and at the fifth active lever-response, mice received another electric foot-shock with a chocolate-flavoured pellet paired with the cue light. The schedule was reinitiated after 10 sec pellet delivery (time-out period) and after the fourth response if mice did not perform the fifth response within a minute. The total number of foot-shocks at fourth (punishment without reward) and fifth response (punishment and reward delivery) to the active lever were registered to evaluate the compulsivity-like behaviour. In this test, each pellet delivered was associated with punishment (foot-shock), previously described as resistance to punishment (Deroche-Gamonet, Belin and Piazza, 2004; Mancino et al., 2015).

Cognitive flexibility: active-lever presses/total of lever presses ratio in the reversal test was used to evaluate the cognitive flexibility. Each animal is

exposed to the FR5 schedule session with inverted active lever presses for 1 h. Both new active and new inactive is recorded for ratio calculation.

Impulsivity: Non-reinforced active responses during the time-out periods (10 sec) after each pellet delivery were measured as impulsivity-like behaviour, indicating the inability to stop a response once initiated (Koob and Volkow, 2009). The last three consecutive days of the self-administration period were considered.

After performing all the behavioural tests, the three main vulnerability food addiction-like behaviour criteria are persistence to response, motivation, and compulsivity. According to them, mice were categorized as vulnerable and non-vulnerable animals depending on the number of positive criteria they had achieved. An animal was considered positive for a vulnerable food addiction-like criterion when the score of the specific behavioural test was above the 75th percentile of the normal distribution of the control group. Mice that achieved two or three food addiction-like criteria were considered vulnerable animals, and mice that achieved 0 or 1 food addiction-like criteria were considered non-vulnerable animals. The rest of the tests were useful for the group phenotypic categorization.

Emotional test

Anxiety-like behaviour

We performed the elevated plus-maze test (EPM) in a black Plexiglas apparatus with two opened (45 lux) and two closed (5 lux) arms (29 cm long x 5 cm wide) set in a cross from a neutral central square (5 × 5 cm) and elevated 40 cm above the floor. The percentage of time spent in the

open arms was determined during 5 mins, as previously reported by (Busquets-Garcia et al., 2011).

Depressive-like behaviour

Forced swimming test (FST) was the paradigm used to determine depressive-like behaviour. Mice were placed in a narrow (17.5 × 12.5 cm) plexiglass cylinder containing water to a depth of 15 cm (22 °C ± 0.2 °C) (Porsolt, Bertin and Jalfre, 1977). Each animal was subjected to forced swimming for 6 min. The first 2 mins were considered as habituation. The total duration of immobility, disregarding small maintenance movements, was measured during the last 4 mins, when mice showed a sufficiently stable immobility level.

Cognitive evaluation

The novel object recognition test (NORT) was performed in a black plexiglass apparatus with two arms in a V shape (V-maze). Two types of memory were evaluated, short-term memory (STM) and long-term memory (LTM). In both cases, three phases of 9 mins were performed. On the first phase or habituation phase, mice recognize and explore the V-maze for 9 mins. Then the training phase is performed 24 h later. Two identical objects (chess pieces) are located at the end of each maze's arm during the training phase. The time spent exploring each object is recorded for 9 mins. For the STM, three hours after the training phase, one of the familiar objects was replaced with a novel object (a different chess piece), and the time spent exploring each object (novel and familiar) was computed (test phase). For the LTM, the test phase is performed 24h later than the training phase. A discrimination index was calculated as the

difference between the times that the animal spent exploring the novel (Tn) and familiar (Tf) object divided by the total time of object exploration: (Tn-Tf)/(Tn + Tf).

Locomotor activity

Locomotion was evaluated in the actimetry boxes (10.8×20.3×18.6 cm, Imetronic, Pessac, France) equipped with infrared sensors to detect locomotor activity and an infrared plane to detect rearings as vertical locomotion activity. Mice were individually placed in the boxes, and the kinetics of the rearing and total activity was recorded in blocks of 15 mins for 3 h. Locomotor studies were performed to evaluate possible side-effects of the drugs bilaterally administered that could represent a bias for interpreting the results

RNA-sequencing

Tissue collection

At the end of the experimental protocol, *D2-Cre:RiboTag* mice were sacrificed by cervical dislocation, and the heads were immersed in liquid nitrogen for 4 sec. The brains were then removed and sectioned on an aluminium block on ice. The whole striatum was extracted as previously described (Puighermanal, Biever and Valjent, 2016). The NAc was isolated from a ~1-mm thick coronal section located between 1.94 and 0.86 mm anterior to bregma and the dorsal striatum between 0.86 and 0.14 mm anterior to bregma as previously described (Biever et al., 2015). The samples were placed in individual tubes to immediately frozen them in liquid nitrogen and store them at −80°C until RNA isolation for the RNA-sequencing.

Polyribosome immunoprecipitation

Polyribosome immunoprecipitation was performed in NAc tissue samples obtained from *D2-Cre:RiboTag* mice as previously described Puighermanal et al., 2017. Total RNA was extracted from ribosome-mRNA complexes using RNeasy Micro kit (Qiagen) followed by column DNAse treatment to remove genomic DNA contamination. The quality and quantity of RNA samples were both assessed using Agilent Bioanalyzer 2100 (Agilent technologies). Three biological replicates, each composed of a pool of 3-4 mice, were used for RNAseq analysis.

Stranded mRNA library preparation and sequencing

According to the manufacturer's protocol, the libraries from the total mouse RNA were prepared using the TruSeq® Stranded mRNA LT Sample Prep Kit (Illumina, Inc., Rev.E, October 2013). Briefly, 0.25 µg of total RNA was used for poly-A-based mRNA enrichment with oligo-dT magnetic beads. The mRNA was fragmented (resulting RNA fragment size was 80-250 nt, with the major peak at 130 nt), and the first-strand cDNA synthesis was done by random hexamers and reverse transcriptase. The second strand cDNA synthesis was performed in the presence of dUTP instead of dTTP; this allowed to achieve the strand specificity. The blunt-ended double-stranded cDNA was 3’ adenylated, and Illumina platform compatible adaptors with unique dual indexes and unique molecular identifiers (Integrated DNA Technologies) were ligated. The ligation product was enriched with 15 PCR cycles, and the final library was validated on an Agilent 2100 Bioanalyzer with the DNA 7500 assay. Each library was sequenced on HiSeq4000 (Illumina) in a fraction of a HiSeq 4000 PE Cluster kit sequencing flow cell lane, following the manufacturer's

protocol for dual indexing. Image analysis, base calling and quality scoring of the run were processed using the manufacturer's software Real-Time Analysis (RTA 2.7.7) and followed by the generation of FASTQ sequence files.

Bioinformatic analysis

We sequenced at least 20 million paired-end reads per sample using the dUTP protocol for strand selection. Reads were mapped using the STAR alignment on the mouse mm10 genome, and mapped reads were counted on gencode vM5 annotation both at the gene and transcript level. The transcriptional variance was visualized using principal component analysis normalizing the data using the DESeq function "variance Stabilizing Transformation". Differential expression analyses were performed using DESeq (gene-level) and DEXseq (transcript level). We calculated two different contrasts: "Binge-eating *vs* Free-choice" and "Binge-eating *vs* Standard food" and considered them as differentially expressed genes with a Benjamini adjusted p-value < 0.05. We performed enrichment analysis using the R package "cluster profile" interrogating the GO, Reactome and KEGG databases. The R package TagCloud and heatmap.2 were used for visualization purposes.

cDNA synthesis and quantitative real-time PCR

We designed real-time PCR primers of the selected genes to evaluate changes in their expression to confirm the results of the RNA sequencing. RNA was reverse transcribed using a high-capacity RNA-to-cDNA kit (Applied Biosystems, 4390778). Primers were designed using Primer3 (v. 0.4.0) and checked using PrimerBLAST, NCBI (National Center for

Biotechnology Information, US National Library of Medicine). We performed real-time qPCR on pull samples in triplicate in 384-well PCR-plates using the QuantStudio™ 12K Flex Real-Time PCR System (Thermo Fisher, Scientific) and SYBR Green (Sigma–Aldrich, St. Louis, MO, USA). The expression of 5 candidate genes (Adoara2a, CCK, DAGLα, GABRG1, D2R) were investigated. Real-time PCR analysis was carried out with the following primers (gene name: F and R sequence):

Adora2a: F' TCGCCATCACCATCAGCACTGG – R' TGATGCCCTTCGCCCTCATACC

CCK: F' ATCCAGCAGGTCCGCAAAG – R' TCCAGGCTCTGCAGGTTCTTA

DAGLα: F' CCACAGAGCATCGCAACAGCAG – R' TGGACAGCAGCAGAAGCTCTACG

GABRG1: F' TGGACCTGTGGATCCCATAAAC – R' TGAGCATAAGTACTTTCATGGTGCTA

D2R: F' AAGACGATGAGCCGCAGGAAGC – R' TGGGGTTCACGGCACTGTTG

USP11: F' AGTGGCGCCAAATCGGGAATG – R' ATCCCCTCCCTTCACGTACACC

We used as an internal control (housekeeping) the ubiquitin-specific peptidase 11 (Usp11) gene because it was highly expressed and less variable across groups. Relative expression of mRNAs was determined after normalization with housekeeping genes using the ΔΔCt method.

Immunofluorescence study

Tissue preparation for immunofluorescence

Mice were deeply anesthetized by i.p. injection (0.2 ml/10 g of body weight) of a mixture of ketamine/medetomidine prior to intracardiac perfusion with 4% paraformaldehyde in 0.1 M Na2HPO4/ 0.1 M NaH2PO4 buffer, pH 7.5, delivered with a peristaltic pump at 30 ml per min for 2 min.

Subsequently, brains were extracted and post-fixed with 4% PFA for 24 h and transferred to a solution of 30% sucrose at 4°C. Coronal frozen sections (30 µm) of the NAc core and VP were obtained on a freezing microtome and stored in a 5% sucrose solution at 4°C until use.

Immunofluorescence protocol

Free-floating slices were rinsed in 0.1 M PB, blocked in a solution containing 3% normal goat serum and 0.3% Triton X-100 in 0.1M PB (NGS-T-PB) at room temperature for 2 h, and incubated overnight at 4°C in the same solution with the primary antibody to anti-Cre recombinase (1:500, mouse, MAB3120, Merck Millipore). On the next day, after 3 rinses in 0.1 M PB, sections were incubated with the secondary antibody AlexaFluor-488 donkey anti-mouse (1:500, Life Technologies) at room temperature in NGS-T-PB for 2 h. After incubation, sections were rinsed and mounted immediately after onto glass slides coated with gelatine in Fluoromount mounting medium.

Image analysis

The stained sections of the brain were analyzed with Leica TCS SP5 CFS (fixed stage) upright confocal microscope with two non-descanned HyD detectors. The images were processed using the ImageJ analysis software.

Experimental design

Study 1: **Endogenous depletion of 2-AG levels in the nucleus accubems reduce palatable food intake in binge-like eating mice**

Study of the transcriptomic profile in NAc D2(+) neurons after exposure to intermittent restricted access to cafeteria diet.

We took advantage of the recently generated *D2-Cre:RiboTag* transgenic mice to evaluate the transcriptomic profile associated with the development of binge-like eating behaviour. This transgenic mouse line allowed the isolation by an immunoprecipitation process of the mRNA actively translated into protein exclusively in D2(+) neurons (Puighermanal et al., 2020). *D2-Cre:RiboTag* mice (n = 36) with similar body weights were divided into three groups according to the diet: (1) Standard group (STD), (2) Free-choice group (FC), and (3) Binge-eating group (BE). The STD group had *ad libitum* access to StdF for the entire experiment, while the FC group were exposed to *ad libitum* free-choice access to StdF and CD for the entire experimental sequence. The animals on the BE group had unlimited access to StdF and were exposed to six BC (24 h/week of free-choice access to StdF and CD). Total food intake from StdF and CD (grams of food) was evaluated at different time points (1.5 h and 24 h from BC onset) together with the body weight during each BC. Mice from all three experimental groups were sacrificed 1.5 h after the exposure to CD of the last BC in BE animals (on week 6). The NAc was extracted and processed to perform the polysome immunoprecipitation and posterior transcriptomic analysis exclusively in NAc D2(+) cells. Results from the RNAsequencing analysis together with bibliography documentation allowed the selection of different candidate genes that were further validated in modulating binge-like eating episodes using a pharmacological approach.

Pharmacological evaluations of the different candidate genes.

Previous to the evaluation in the BE model, a preliminary study was performed to investigate possible undesired side-effects of the different doses of each compound that underwent bilaterally intra-NAc

administrations. For this purpose, 31 WT C57BL/6J male mice were bilaterally implanted into the NAc. Animals were divided in different groups according to the compound administered: (1) Control groups: Saline (n = 6); and 5% DMSO in Saline (n = 10); (2) Drug groups: CCK-8S (n = 5); CGS-21680 (n = 5) and O-7460 (n = 10). The effects of the different doses of each compound administered intra-NAc were evaluated in the Irwin test, locomotion and anxiety paradigms. A 7-days wash-out period was left in between each intra-NAc administrations and test performance.

These compounds were also evaluated in the model of binge-like eating behaviour to validate the involvement of each candidate gene selected in regulating compulsive overeating. A total of 64 male mice C57BL/6J underwent the same surgical procedure to get bilateral cannula implantation into the NAc and were divided into two experimental groups according to diet: (1) STD group (n = 30) and (2) BE group (n = 34). At the beginning of the experiment, mice were exposed to two intra-NAc vehicle administrations. Food intake was measured to obtain basal food intake values on both diet groups. These basal intake after vehicle administration was used to homogeneously divide the animals into the following groups: (1) mice exposed to STD that received intra-NAc CCK-8S (n = 8); CGS-21680 (n =10) or O-7460 (n = 12); and (2) mice exposed to BE that received CCK-8S (n = 8); CGS-21680 (n = 15) or O-7460 (n = 11). Then, the basal phenotype was compared to the binge-eating episode performance after intra-NAc drug injection. Different doses of each drug were evaluated. Intra-NAc injections were performed for 3 min (2 minutes administration and one extra minute post-injection to assure no-reflow) into their home cages immediately before the exposure to CD on each BC. Food intake (StdF and CD) and body weight were measured after 1.5 h, 3 h, and 24 h

post-injection. A 7-days wash-out period was left in between each intra-NAc administrations and test performance.

Study 2: Adenosine A_{2a} receptor overexpression into the nucleus accumbens to ventral pallidum pathway decreases motivation for chocolate in binge-like eating mice

Accumbal $A_{2A}R$ overexpression in ventral striatum indirect pathway

The protocol was divided in two phases: training phase and experimental phase. In the training phase, WT mice (n = 50) with *ad libitum* access to StdF and water were trained to acquire the operant conditioning maintained by chocolate-flavored pellets under FR1 (5 sessions) and FR5 (7 sessions) schedule of reinforcement. Once the criteria for the achievement the operant responding were acquired, the number of active-lever presses during the last 3 sessions of FR5 schedule, together with the body weight were the criteria to mice group division. Before surgeries, the total 50 mice were divided into four homogeneous groups according to the diet and operant responding performance: AAV-CTRL-STD (n=10); AAV-A2aR-STD(n=11); AAV-CTRL-BE (n=15); AAV-A2aR-BE (n=13). Then, one week before surgeries, animals were exposed to pre-surgery BC to obtain basaline data of feeding pattern in all groups (BE and STD groups). Later, all animals underwent to a bilateral intracranial microinjection of the AAV8-hSyn-DIO-Adora2a-mCherry (AAV-A2a) or AAV8-hSyn-DIO-mCherry (AAV-CTRL) in the NAc core together with bilateral intracranial microinjection of the AAVrg pmSyn1-EBFP-Cre in the VP. One week was considered for full recovery from surgeries. Following surgeries, animals were exposed to BC once a week for six weeks to develop the binge-like eating feeding pattern in BE groups (see section

behavioral experiments; model of binge-like eating behaviour). The total expression of the AAVs was considered after 4 weeks from biltateral microinjection. Thus, to evaluate the effect of AAV-injection in binge-like eating episodes regulation, BCs after BC4 were considered to obtain relative differences in the escalation of compulsive overeating by comparing AAV-injected groups. BCs performed in combination to operant self-administration protocol were excluded to avoid feeding pattern interferences produced from chocolate-flavored pellets obtained in each operant session. The relative difference of food intake for both StdF and CD was calculated comparing the difference of BC from basaline conditions (before surgeries) and subsequent BC in experimental phase to their average magnitude $[(X-X_{ref})/((X+X_{ref})/2)]$.

To determine phenotypical traits between groups, at the week 7^{th} the model of binge-like eating behaviour protocol was combined with the self-administration protocol. Voluntary binge-eating protocol was performed during two days at the beginning of the week, and the self-administration protocol the following three days. Both protocols were not mixed in the same day. The motivation, cognitive flexibility and compulsivity tests were performed at different weeks following the three day of standard self-administration protocol. Emotional and cognitive test were performed in different weeks together with voluntary binge-eating behavioural protocol once the operant self-administration protocol was finished. To verify viral expression, mice were perfused at the end of the experiment (16^{th} week) and the fluorescent reporter mCherry was visualized in brain slices. Cre-recombinase expression was detected by immunofluorescence using an anti-Cre recombinase antibody (see section "Immunofluorescence study").

Study 3: Behavioural characterization of a mouse model of binge-like eating behaviour in PheComp cages

A longitudinal model of binge-like eating behaviour

Animals were individually housed in PheCOMP multi-take metabolism and activity cages (Panlab-Harvard Instruments, Barcelona, Spain) to obtain detailed feeding behaviour data (full description of the PheCOMP cages in Bura et al., 2010). Two food dispensers record the feeding data. Similar to the model of binge-like eating behaviour protocol described above, animals were fed with StdF (with StdF in both food dispensers) during the entire experimental period and were exposed to BC once a week (with StdF in one food dispenser and CD in the other) for a total of 7 weeks. The meal pattern of StdF and CD was first processed using R scripts (R Core Team 2015) and further analyzed using the COMPULSE software (Panlab-Harvard Instruments, Barcelona, Spain) and Pergola web-server to process and visualize longitudinal behavioural data (Espinosa-Carrasco et al., 2018; Fructuoso et al., 2019). We analyzed the feeding pattern before, during and after CD exposition by recording the number of meals (episodes-related frequency after 1.5 h, 3 h and 24 h during the BC), the average duration of meals (intake velocity), the total energy consumed and food consumed per second to obtain the eating rate (binge-eating episode) and satiety rate. These measures allowed us to create corresponding criteria in a mouse model of binge-eating disorder based on the diagnosis criteria described in DSM-5 for such disorder (American Psychiatric Association, 2013). Animals were recorded uninterruptedly during periods of 3 or 4 days. The system was paused for approximately 1 h between periods for refilling the feeders and cleaning the cages

Statistical analysis

All statistical comparisons were performed with SPSS (IBM, version 25). Comparisons between two groups were analysed by Student t-test or U Mann–Whitney and within groups by paired t-test or Wilcoxon test depending on the distribution defined by the Kolmogorov-Smirnov normality test. In case of more than two independent groups, one-way ANOVA was used. ANOVA with repeated measures was used when the same animals were exposed to more than two conditions or to test the evolution over time. Mix model test was used to compare more than two groups with repeated measures on the same animal. Additionally, Bonferroni's multiple comparison test was used to compare two groups in each time point or measure repetition. Results were expressed as individual values with the median and the interquartile range or the mean ± SEM specified in the figure legend. A p-value < 0.05 was used to determine statistical significance. The sample size was calculated based on the power analysis. The significance (alpha) criterion was set at 0.050, and the statistical test used was a two-sample t-test. With the sample size of 8-15 mice per group, our studies achieved a power between 73 and 90%. Supplementary tables provided a complete report of the statistical results for the data described in the figures.

Results

Study 1

A Gallego-Román[1], I. De Toma[2,4], E. Puighermanal[3, 5], M. Dierssen[1,2,4], E. Valjent[5*], R. Maldonado[1,2*], E. Martín-García[1,3*], M. Martín-Sánchez[1#*].

[1]. University Pompeu Fabra, Barcelona, Spain.

[2]. IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain.

[3]. Universitat Autònoma de Barcelona, Bellaterra, Spain.

[4]. Centre for Genomic Regulation (CRG), Barcelona, Spain.

[5]. IGF, CNRS, INSERM, Université Montpellier, Montpellier, France.

[#] Current address: Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain.

[*] Equally contributed.

Under preparation

Endogenous depletion of 2-AG levels in the nucleus accumbens reduce palatable food intake in binge-like eating mice

Intermittent access to cafeteria diet leads to the development of binge-like eating behaviour

Male D2-Cre:RiboTag mice (Puighermanal et al., 2020) were divided into three experimental groups according to the diet (standard, STD; free-choice, FC and binge-like eating, BE) and exposed to the experimental protocol depicted in Figure 24a. Our results showed that mice with intermittent access to cafeteria diet (CD) once a week for a 24 h period (BE experimental group) significantly increased the energy intake (kCal/gr animal) in the first 1.5 h from the binge-like eating cycle (BC) onset compared to STD and FC animals (diet_group $F(2, 31) = 6.439$; $p< 0.01$.Tuckey's posthoc STD vs FC n.s; STD vs BE $p<0.05$; BE vs FC $p<0.01$) (Figure 24b). During this period, mice within the BE experimental group displayed the principal phenotypic trait of binge-eating disorder (BED), consuming large amounts of food in a short period of time (around 15% of their regular total daily caloric intake in 1.5 h). Repeated measures ANOVA analysis revealed that this aberrant feeding behaviour in BE mice was due to a significant increase in the energy consumed from CD when compared to FC animals (group*diet $F(1, 32)= 21.830$, $p<0.001$) (Figure 24c). At the end of each BC (following 24 h from CD exposure), the total energy intake significantly differed among experimental groups, with the BE mice showing the highest consumption ($F(2, 31)= 22.31$, $p<0.001$, Tuckey's posthoc STD vs FC $p<0.001$; STD vs BE $p<0.001$; BE vs FC $p<0.05$) (Figure 24d). However, statistical analysis did not reveal differences in the CD

intake between BE and FC animals during each BC at the end of the BC (group*diet $F_{(1, 32)}$= 21.830, n.s) (**Figure 24e**). On the other hand, there was no evidence of habituation or loss of motivation to consume CD in BE mice after prolonged exposition to repetitive binge eating cycles (**Figure 24c**). Differences in body weight were also detected among groups. Mice fed with *ad libitum* free-choice access (obese-like fed conditions) significantly increased their body weight at the end of the protocol compared to BE and STD mice. However, no significant alterations were observed when comparing the BE to the STD group (**Figure S1**).

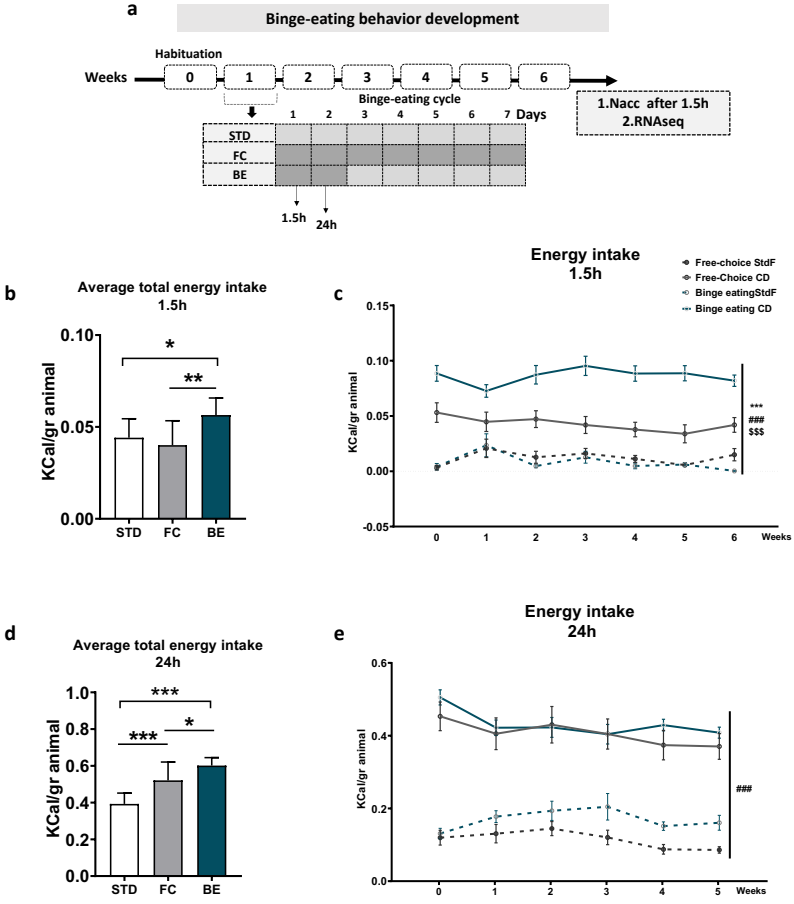


Figure 24. Intermittent access to CD produces BE episodes. a) Timeline of the experimental protocol used to induce BE behaviour. D2-Cre:RiboTag mice (n=36) were exposed to three different diet conditions: STD group (n=12) with *ad libitum* access to StdF; FC group (n=12) with *ad libitum* free-choice access to both StdF and CD; and BE group (n=12) with 24 h period/week with *ad libitum* free-choice access to both StdF and CD. Light grey represents *ad libitum* access to StdF, and dark grey the free-choice access period to both, StdF and CD for each experimental group. b) Average total energy intake after 1.5 h from BC onset in kCal/gr animal on each experimental group (STD, FC and BE). c) Energy intake on each BC from StdF and CD after 1.5 h exposure to CD on FC and BE groups. d) Average total energy intake after 24 h from BC onset in kCal/gr animal on each experimental group (STD, FC, and BE). e) Energy intake on each BC from StdF and CD after 24 h exposure to CD on FC and BE groups. Differences between groups disappear after 24 h. Average total energy intake was analysed by Tuckey's posthoc following a one-way ANOVA test. *p<0.05, **p<0.01, ***p<0.001. Energy intake on each BC was analysed by a repeated-measures ANOVA test. group***p<0.001; diet ###p<0.001; group*diet $$$p<0.001. All data are shown as Mean ± SEM. Statistical details are included in supplementary table S1.

Binge-like eating behaviour is associated with transcriptomic alterations in NAc D2(+) neurons

Changes in gene expression in NAc D2(+) neurons were evaluated 1.5 h after the onset of the last BC when compulsive overeating behaviour was evident. First, overall transcriptional changes between diet groups were determined by applying a principal component analysis (PCA). In this analysis, the repetitive binge eating by intermittent free-choice access to CD was the main source of variance (61%), with BE mice differing from those in FC and STD groups as revealed by the different cluster distributions observed in the plot (**Figure 25a**). In accordance, RNA sequencing analysis showed similar significant changes in the gene expression in BE mice compared to both STD and FC animals (**Figure 25b and Figure S2a**). Differential expression analysis revealed that from over

20.000 genes expressed in D2(+) neurons, 498 (2.3%) were significantly (FDR<0.05) upregulated in the BE group compared to the STD group, while 396 genes (1.8%) were downregulated (**Figure 25b**). On the other hand, 744 genes (3.4%) were significantly upregulated, and 750 downregulated (3.4%) when BE and FC groups were compared (**Figure S2a**). Interestingly, genes previously related to the reward system such as *Drd2* (dopamine receptor type 2), *Adora2a* (adenosine receptor 2a), *Cck* (Cholecystokinin), and *Mgll* (Monoglyceride lipase enzyme) mRNA were found to be significantly upregulated in the binge-like eating mice. On the other hand, genes such as the *Gabrg1* (gamma-aminobutyric acid receptor subunit gamma-1) and *Ndn* (necdin protein) were downregulated, suggesting changes in GABA-mediated signalling and genetic disorders that cause hyperphagia.

To further characterise the functional role of the differentially expressed genes (DEGs) in NAc D2(+) neurons in BE mice relative to STD and FC mice, we performed a gene ontology (GO) term enrichment analysis and gene-concept network analysis (**Figure 25c-f and Figure S3 and S4**). The GO analysis revealed a potentiation in the expression of genes involved in different neurobiological processes, highlighting pathways associated with memory, cognition, and learning (**Figure 25c and Figure S3a**) and brain-related cellular components (**see Figure S5**) in both comparisons (BE *vs* FC and BE *vs* STD). Interestingly, high enriched scores in the addiction-related pathway were detected when comparing BE with STD (**Figure 25d**) and BE with FC (**Figure S3b**). The cnetplots (**Figure 25e and f**) depicted the linkages of genes and biological concepts as a network to show the essential terms where the genes differentially expressed were involved. A quantitative real-time PCR (qPCR) was performed to compare

relative differences among dietary groups and validate the RNA sequencing results. The *Cck* transcript levels were increased in BE mice compared to STD and FC mice, but there were no significant differences between STD and FC mice (BE vs STD: t= 4.856 p<0.001; and BE vs FC: t= 4.157, p<0.001). On the other hand, the qPCR analysis confirmed a significant *Drd2* gene expression in mice that binge eat in comparison to those with obese-like fed conditions (FC) (t= 2.787, p<0.05). The transcriptomic analysis revealed a significant increase in *Mgll* transcript levels in BE mice compared to FC and STD mice. Thus, we validate the *Dagla* gene expression to determine a possible 2-AG upregulation during compulsive overeating that may hyperactivate the endocannabinoid system. No significant changes in gene expression were detected when *Dagla* transcript levels were compared among dietary groups (BE vs STD: *t*= 0.3656, n.s; BE vs FC: *t*= 0.5880, n.s). According to our results from the transcriptomic analysis, we selected three candidate targets that play an important role in regulating eating-related behaviours and food intake, as well as addiction-related behaviours, to evaluate their potential therapeutic function in reducing the compulsive-like eating behaviour by modulating the NAc neurons activity: CCK receptors, the endogenous regulation of the endocannabinoid system (ECS) and the adenosine A_{2A} receptor.

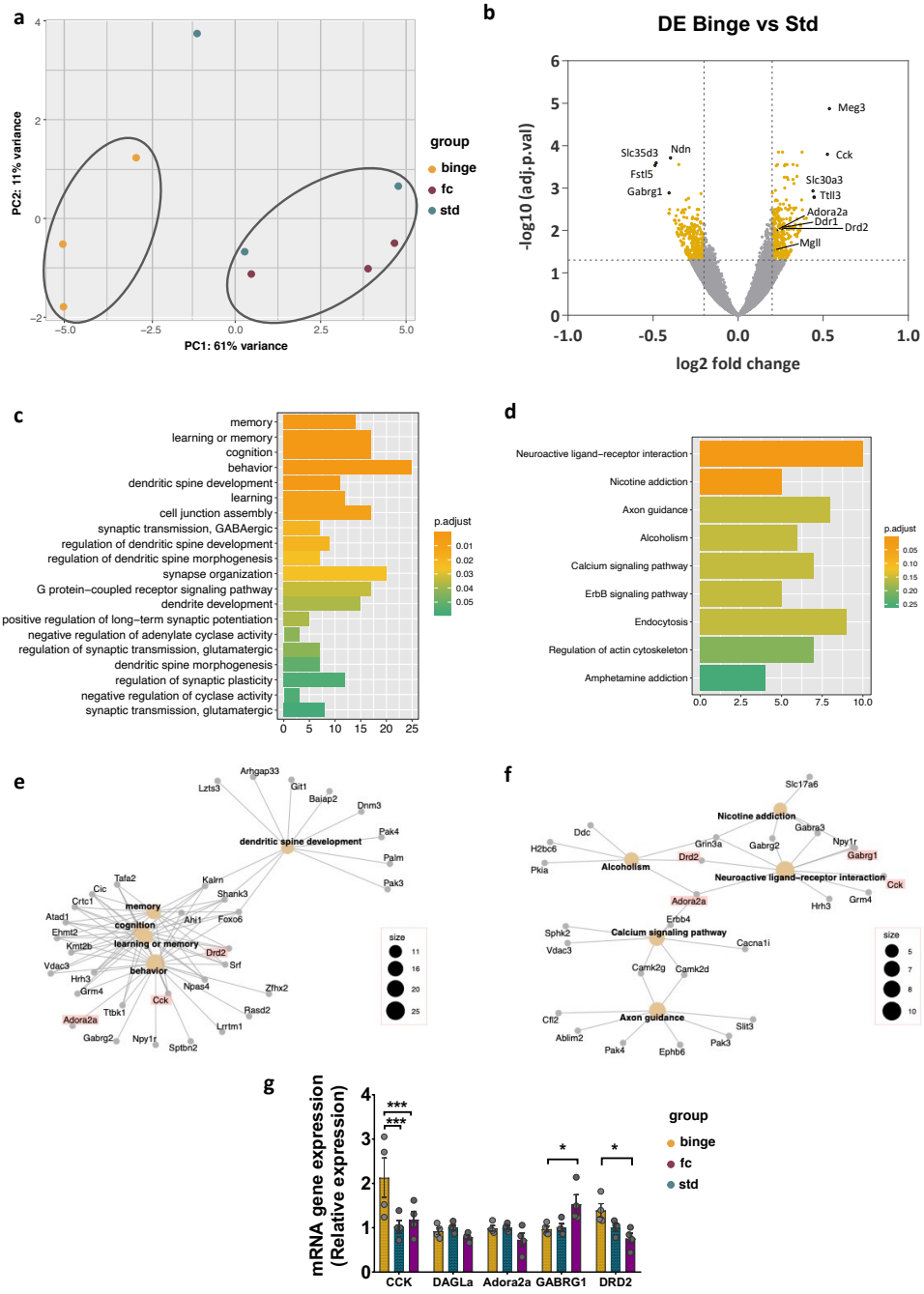


Figure 25. Exposure to a model of BE produces important transcriptomic alterations in NAc D2(+) neurons. a) Principal component analysis. Biplot showing PC1 on the x-axis and PC2 on the y-axis. Each dot represents a biological

replicate, where the BE group is in orange, the FC group in purple and the STD group in blue. The percentage of variance explained for each principal component is indicated. **b)** Volcano plot of the RNA-seq data analysis. Differentially expressed genes in BE mice group compared to the STD mice group. The cut-off of 1.2-fold change and differentially expressed genes (padj <0.05) are highlighted in orange. **c-d)** The colour gradient indicates the adjusted p-value for the enrichment. Numbers in parentheses indicate the number of identified genes in each category. Bar size corresponds to gene count for each group)/(total gene count for each category. **c)** biological processes where are involved DEGs comparing BE vs STD mice. **d)** Addiction-related pathways where are involved DEGs comparing BE vs STD mice. The interrogated databases were KEGG. **e-f)** Gene ontology term enrichment cnetplot analysis of DEGs. The brown nodes denote key pathways. The size of the nodes reflects the number of DEGs. Enrichment analysis was performed with a significant FDR < 0.05. **e)** biological processes where are involved DEGs comparing BE mice with STD mice. **f)** Addiction-related pathways where are involved DEGs comparing BE vs STD mice. Contrast for BE vs FC is shown in the supplementary section. **g)** Quantitative real-time PCR of selected genes (STD n= 4; BE n= 4; FC n=4) (Bonferroni's multiple comparison. *p<0.05, ***p<0.001). Statistical data details are included in supplementary table S2.

Intra-NAc injection of CGS 21680 decrease locomotor activity

A preliminary study was carried out to evaluate possible side-effects from bilateral administration of each compound into the NAc, leading to misleading interpretations. Following the local infusion of each drug, the observational screening test (Irwin test), locomotor activity and anxiety traits were evaluated in an independent batch of mice. A 7-days wash-out period among each intra-NAc administrations and test performance was left, as represented the **Figure 26a**.

On the one hand, to investigate the role of CCK receptors in the NAc in modulating the palatable food intake during binge-eating episodes, the CCK_A and CCK_B receptors agonist, CCK-8S (CCK octapeptide sulphated), was bilaterally administered into the NAc at 2ng/side dose. Immediately

after intra-NAc drug administration, behavioural responses were evaluated in the Irwin test for drug screening for 30 min. No adverse side-effects were detected by observational screening (battery of test compiled in **Supplementary Table 3**). One week later, the possible locomotor activity effect following NAc CCK receptors activation was evaluated for 3 h immediately after bilateral intra-NAc administration of CCK-8S (**Figure 26b**). No alterations in horizontal (CCK-8S: $F_{(11, 198)}$=0.810, n.s) or vertical (CCK-8S: t= 1.199, n.s) locomotor activity were observed in mice exposed to intra-NAc CCK-8S infusion in comparison to the control group (**Figure 26b and e**). The anxiety-like behaviour was evaluated using the elevated plus-maze test (EPM) following one week from the locomotion test. We did not observe alterations in the percentage of time spent in the open arms in the animals injected with CCK-8S via intra-NAc when compared with the control vehicle group (CCK-8S: t=0.757, n.s).

On the other hand, a selective agonist for the adenosine A_{2A} receptor, CGS 21680, was used at different doses to local activate the A_{2A}R in the NAc by bilateral administration of two different doses, 1ng/side and 2ng/side. No side-effects were reported during the Irwin test for drug screening for 30 min by observational screening at any dose. However, we detected a significant decrease in the total locomotor activity during the first 75 min in animals treated with 1ng/side dose of CGS 21680 (**Figure S9**) and 120 min in those treated with 2ng/side dose ($F_{(11,110)}$= 3.155, $p<0.05$) (**Figure 26c**), relative to the control group. This effect disappeared in the last 105 and 60 min of the test, respectively. No changes in the total number of rearings representing vertical activity were observed with this compound, 2ng/side of CGS 21680 (t= 2.125, n.s) (**Figure 26f**). In addition, no significant changes in the percentage of time spent in the open arms as

representative anxiogenic-like behaviour were obtained (CGS 21680: t= 3.039, n.s) (**Figure 26i**).

Regard the endogenous regulation of the ECS activity, specific selective inhibition of 2-AG biosynthesis via DAGLα activity inhibition was used to discern the NAc 2-AG reduction role in overeating regulation. One dose of O-7460 (10ng/side) was bilaterally administered into the NAc immediately before the behavioural testing. No significant differences in comparison with the control group injected with 5% DMSO+saline were observed in any test: Irwin, locomotion (horizontal activity: $F_{(11, 110)}$=1.500, n.s and vertical activity: t= -1.936, n.s) and anxiety test (U=27.00, n.s) (**Figure 26d, g and j**).

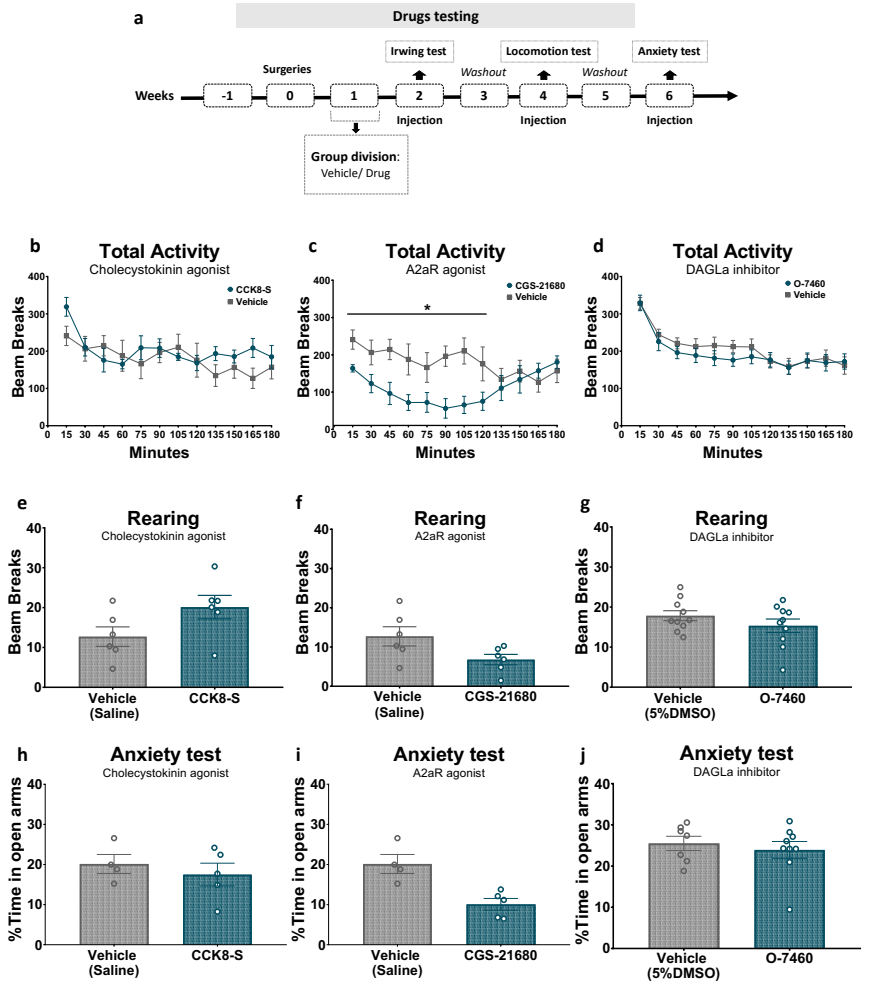


Figure 26. CGS 21680 injection into the NAc alters locomotor activity. a) Timeline of the drug testing. b-d) total activity in beam breaks for 3 hours (repeated measures ANOVA test drug*time *p<0.05. mean ± SEM). e-g) Rearing activity in beam breaks for 3 hours (t-test. mean ± SEM). h-j) Anxiety results by plus-maze test (t-test and U Mann-Whitney test. mean ± SEM). Vehicle (saline) n= 6; Vehicle (saline + 5% DMSO) n= 10; CCK-8S and CGS 21680 n= 6; O-7460 n= 10. All data showed the higher dose used in the following pharmacological validation (CCK-8S: 2ng/side; CGS 21680: 2ng/side; O-7460: 10ng/side). Statistical details are included in supplementary table S4.

Endogenous depletion of 2-AG levels in the NAc by O-7460 infusion modifies the energy intake pattern in BE mice

Next, we evaluated the effect of the different compounds on the regulation of binge-like eating episodes. We performed a pharmacological validation by a longitudinal study following the protocol depicted in **Figure 27a**. To study the feeding pattern depending on the drug dose, mice were divided into two groups of diet: STD (control-diet group) and BE (intermittent free-choice access to CD group). The basal energy intake for both diets groups was established by measuring the kCal/gr animal at different time points following local vehicle infusion: 1.5 h, 3 h and 24 h.

Regard the CCK receptors local activation, the intra-NAc administration of CCK-8S did not modify the total energy intake after 1.5 h compared to their basal conditions in any group of diet, independently of the dose and type of food compared to the control-injection group (STD group: $\chi^2_{(2)}$= 2.138, n.s; BE group: StdF $\chi^2_{(2)}$= 0.875, n.s, CD $\chi^2_{(2)}$= 2.385, n.s) (**Figure 27c**). Gene expression alterations were evaluated 1.5 h after the onset of the BC when BE behaviour was relevant. An additional 3 h time point was included in the pharmacological studies to ascertain possible long-term effects of the tested compounds on energy intake during binge eating. Similarly to the effects previously observed during the first 1.5 h, the CCK-8S intra-NAc injection did not modify the total energy intake at any dose in any group of diet, relative to baseline (STD group: StdF $F_{(2,14)}$= 1.273, n.s; BE group: StdF $F_{(2,14)}$= 0.058, n.s, CD $F_{(2,14)}$= 0.394, n.s) (**Figure 27f**). To evaluate the long-lasting effects of the intra-NAc drug injections, the total energy intake was measured 24 h from the BC onset and drug infusion. The activation of NAc CCK receptors by CCK-8S did not cause significant modification in the amount of energy consumed,

independently of the type of food, in any experimental group of diet at any dose in comparison to the control-injection group (STD group: StdF $F_{(2,14)}$= 0.721, n.s; BE group: StdF $F_{(2,14)}$= 0.007, n.s, CD $F_{(2,14)}$= 1.242 n.s) (**Figure 27i**).

The local activation of NAc adenosine A_{2A} receptors by pharmacological validation revealed an effect of CGS 21680 injection via intra-NAc in reducing the total kCal/gr animal consumed from the palatable food (CD $\chi^2_{(2)}$= 11.828, p<0.01), with no effect in the standard food intake in the BE group (StdF $\chi^2_{(2)}$= 0.255, n.s). This injection effect was detected at 2ng/side dose (vehicle. *vs* high dose Z= -3.078, p<0,01), but not at 1ng/side dose compared to the control group with vehicle injection (vehicle *vs* low dose Z= -1.163, n.s). Concerning the control-diet group of this injection, no changes were observed in the amount of kCal consumed from the standard food at any dose following 1.5 h from the local infusion and high palatable food presentation (StdF $\chi^2_{(2)}$= 3.211, n.s) (**Figure 27d**). Furthermore, the NAc A_{2A}R activation decreased the amount of kCal/gr animal from CD following the highest dose of injection (2ng/side) (CD $\chi^2_{(2)}$= 14.400, p<0.01, veh. *vs* high_dose z= -2.953, p<0.01), without significant changes in the amount of energy consumed from the standard food, following 3 h from the local infusion and cafeteria diet presentation (StdF $\chi^2_{(2)}$= 0.255, n.s). Notwithstanding, we also observed a significant decrease in the total kCal consumed from standard food in the diet-control mice injected with 2ng/side of CGS 21680 via intra-NAc, in comparison with baseline conditions (StdF $\chi^2_{(2)}$= 11.697, p<0.01; veh. vs high_dose z= -2.675, p<0.01) (**Figure 27g**). Interestingly, the suppressive effect of 2ng/side of CGS 21680 injection in palatable food intake was maintained in those mice belonging to the BE group following 24 h from the local

infusion and cafeteria diet presentation (CD $\chi^2_{(2)}$= 8.400, p<0.05). This feeding suppression, however, was not detected at lower doses (1ng/side) (vehicle vs low dose, z= -1.223, n.s) neither in the standard food intake in both groups of diet (STD group: $X^2_{(2)}$= 2.400, n.s; BE group $X^2_{(2)}$= 4.508, n.s) (**Figure 27j**).

On the other hand, the endogenous depletion of NAc 2-AG by inhibiting DAGlα activity showed no significant changes in the energy intake from CD (*t*= -0.843, n.s), but a significant increase in the total amount of energy consumed from standard food in those mice exposed to free-choice conditions, following 1.5 h from 10ng/side dose infusion of O-7460 via intra-NAc (*t*= 2.264, p<0.05) (**Figure 27e**). This significant effect was not detected in mice exposed to *ad libitum* access to standard food compared to the control-injection group (t= 1.353, n.s). The O-7460 injection into the NAc produced a delayed suppression of palatable food intake detected by the significant reduction in the total energy intake from CD (t= -2.706, p<0.01), without significant changes in the kCal consumed from standard food (*t*= 1.121, n.s) in mice exposed to intermitter free-choice access (BE group), relative to the control-injection group (**Figure 27h**). Meanwhile, no changes were observed in the diet-control mice (STD group) that received the same injection dose (10ng/side) in comparison to the baseline conditions (StdF *t*= -1.740, n.s). Interestingly, the reduction of 2-AG in the NAc by 10ng/side of O-7460 injection had a prolonged effect in feeding regulation, decreasing the total amount of kCal/gr animal from CD (*t*= -2.293, p<0.05) and increasing the standard food intake (*t*= 2.269, p<0.05) to compensate the total daily caloric intake in 24 h (**Figure 27k**). No significant changes in the total intake were observed in the control group (STD group) comparing groups of injection (*t*= 0.591; n.s).

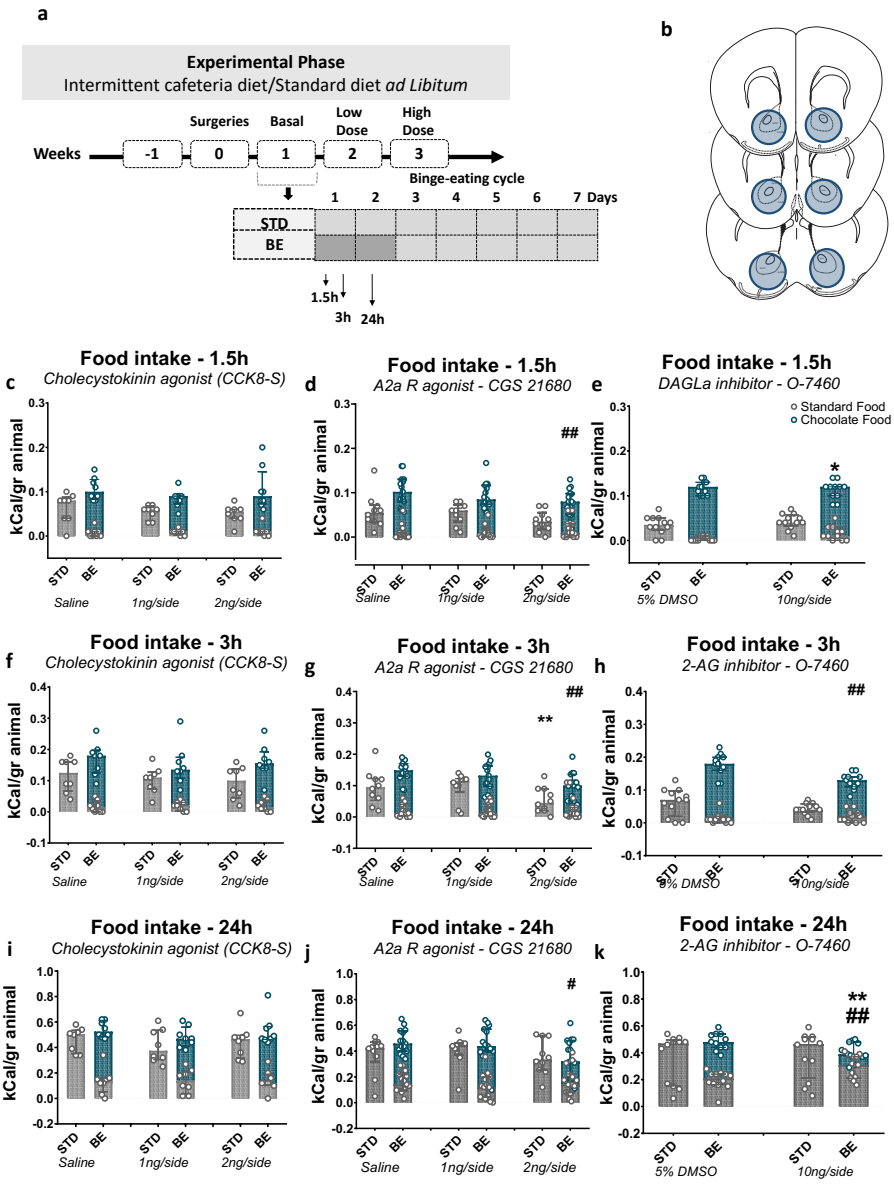


Figure 27. Endogenous regulation of 2-AG levels reduces CD intake following 3 h and 24 h. a) Timeline of the pharmacological validation protocol. Mice were habituated to the BE protocol after intra-NAc injection by vehicle injections after surgeries (basal feeding pattern). Later, two different doses of the drug were tested. Light grey represents ad libitum access to StdF, and dark grey the intermittent free-choice access to StdF and CD. b) Schematic representation to

highlight the delimited area for acceptance of cannula placement in the NAc after methylene blue injection. c-e) Food intake results (StdF and CD) in kCal/gr animal for the first 1.5 h. f-h) Food intake results (StdF and CD) in kCal/gr animal for the first 3h. i-k) Food intake results (StdF and CD) in kCal/gr animal for first 24h. Individual values with the median and the interquartile range. Wilcoxon test and repeated measures ANOVA. StdF: *p<0.05; **p<0.01; CD: ##p<0.01. STD n= 8, 10 and 12; BE n= 8, 11 and 15. Statistical details are included in supplementary table S5.

Supplementary

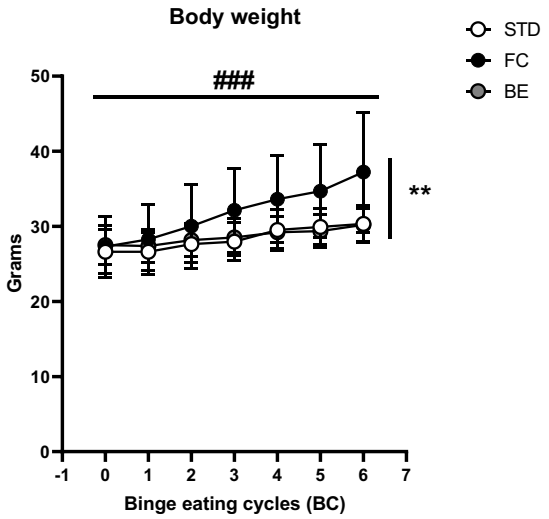


Figure S1. Body weights of the animals throughout the different binge cycles (BC). Mice fed with free-choice access to StdF and CD significantly increased body weight compared to BE and STD mice. (Repeated measure ANOVA. Group*time **p<0.001; time ###p<0.0001. Mean ± SEM). Statistical data detail details are included in supplementary table S2.

Table S1. Model of binge-like eating behaviour (Fig. 24)

Figure number	Statistical analysis	Factor name	Statistic value	P-value
Fig. 24b and d	One way ANOVA	**Total intake 1.5h**	$F(2, 31) = 6.439$	p<0.01
		Total intake 24h	$F(2, 31) = 22.31$	p<0.001
	Tuckey's Post-hoc	**1.5h**		
		STD vs. FC	q= 1.175	n.s
		STD vs. BE	q= 3.590	p<0.05
		FC vs. BE	q= 4.883	p<0.01
		24h		
		STD vs. FC	q= 5.766	p<0.001
		STD vs. BE	q= 9.395	p<0.001
		FC vs. BE	q= 3.720	p<0.05
Fig. 24c and e	Two way ANOVA	**Food intake 1.5h**		
		Group	$F(1,32)= 25.776$	p<0.001
		Diet	$F(1,32)= 148.600$	p<0.001
		Group*Diet	$F(1,32)= 21.830$	p<0.001
		Food intake 24h		
		Group	$F(1,32)= 3.1569$	n.s
		Diet	$F(1,32)= 149.537$	p<0.001
		Group*Diet	$F(1,32)= 0.420$	n.s

Table S2. Model of binge-like eating behaviour and qPCR. (Fig. S1 and 25)				
Figure number	**Statistical analysis**	**Factor name**	**Statistic value**	**P-value**
Fig. S1	**Repeated measures ANOVA test**	**Bodyweight** Group Time Group*time	 F (2, 18)= 1.937 F (1.363, 24,53) = 55.56 F (18, 108)= 61.69	 n.s p<0.001 p<0.001
	Tuckey's post-hoc	STD vs. FC STD vs. BE FC vs. BE	q= 4.982 q= 0.6679 q= 4.751	p<0.01 n.s p<0.01
Fig. 25g	**Bonferroni's multiple comparisons**	**qPCR validation** **BE vs STD** CCK DAGLa Adora2a GABRG1 DRD2 **BE vs FC** CCK DAGLa Adora2a GABRG1 DRD2	 *t*= 4.856 *t*= 0.3656 *t*= 0.0622 *t*= 0.1742 *t*= 1.670 *t*= 4.157 *t*= 0.5880 *t*= 1.142 *t*= 2.480 *t*= 2.787	 p<0.001 n.s n.s n.s n.s p<0.001 n.s n.s p<0.05 p<0.05

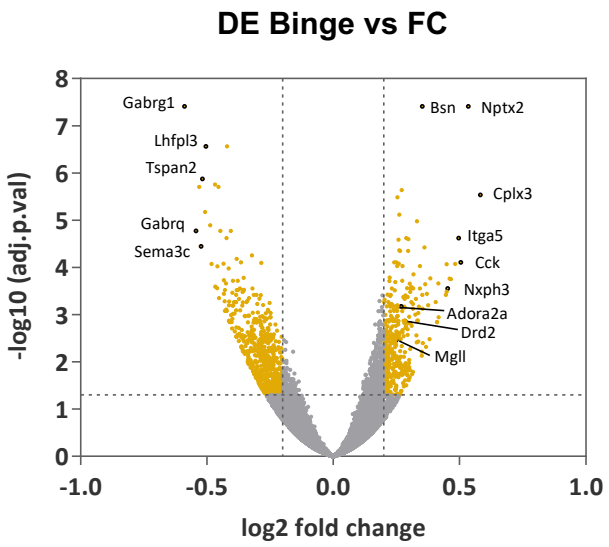


Figure S2. Differentially expressed genes in BE mice group compared to the FC mice group. Volcano plot of the RNA-seq data analysis. The cut-off of 1.2-fold change and differentially expressed genes (padj< 0.05) are highlighted in orange.

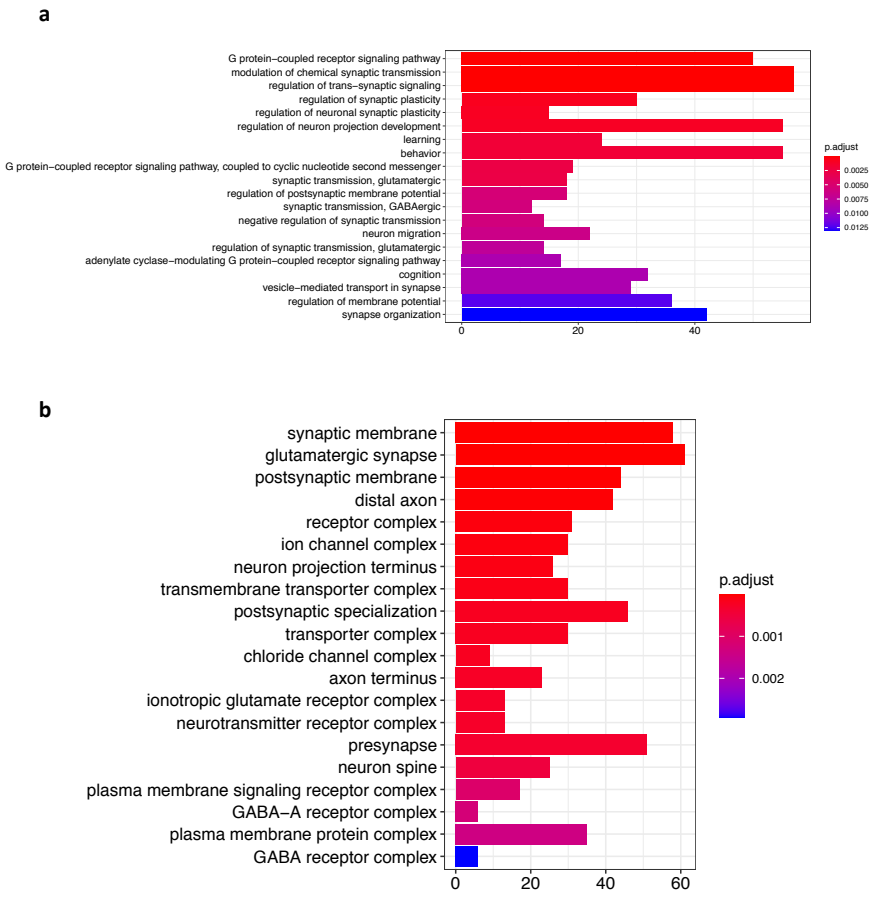


Figure S3. Barplots showing the enrichment analysis of DEGs. a-b) The colour gradient indicates the adjusted p-value for the enrichment. Numbers in parentheses indicate the number of identified genes in each category. Bar size corresponds to gene count for each group)/(total gene count for each category. a) Biological process where are involved DEGs comparing BE vs FC mice. b) Addiction-related pathways where are involved DEGs comparing BE vs FC mice. The interrogated databases were KEGG.

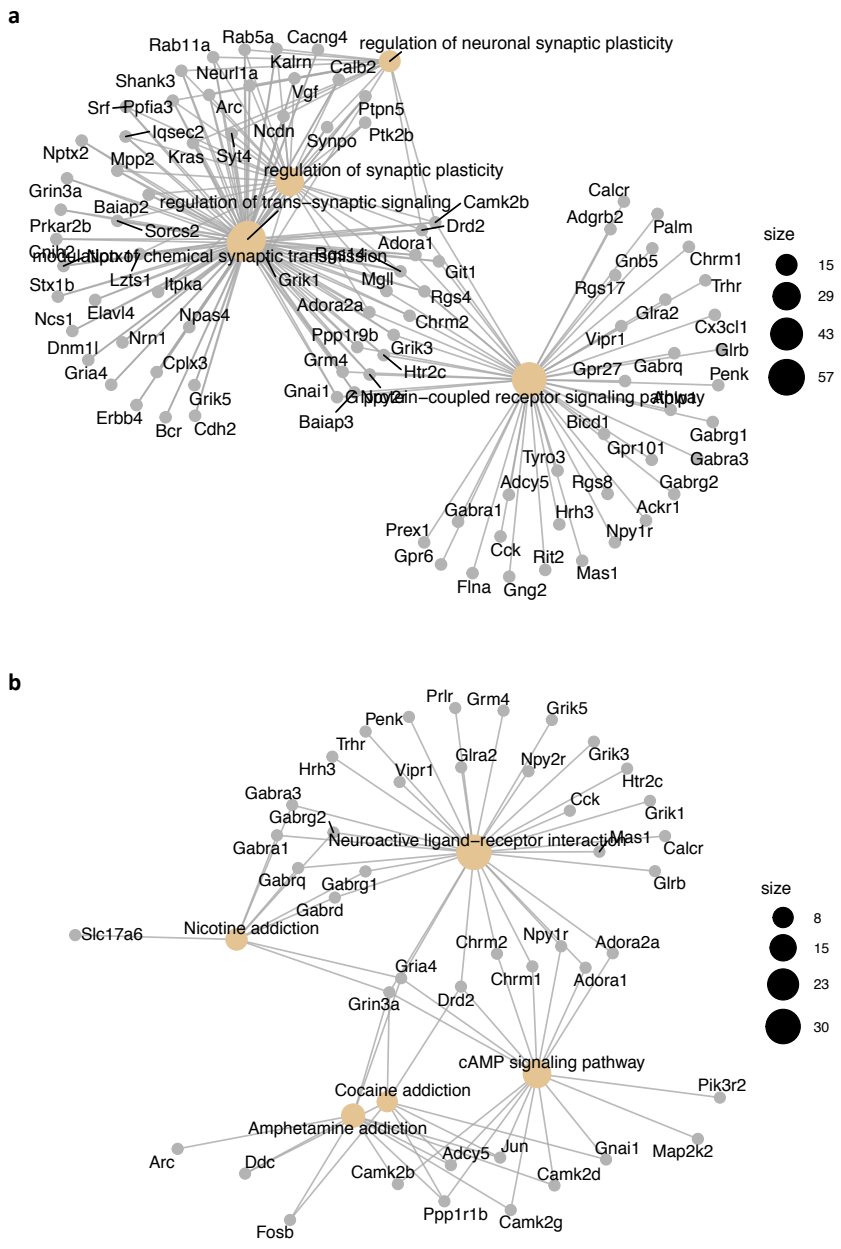


Figure S4. cnetplots showing the enrichment analysis of DEGs. Gene ontology term enrichment cnetplot analysis of DEGs. The brown nodes denote key pathways. The size of the nodes reflects the number of DEGs. Enrichment analysis was performed with a significant FDR < 0.05. a) Biological processes pathways cneplot comparing BE mice with FC mice. b) Addiction-related pathways cneplot comparing BE mice with FC mice.

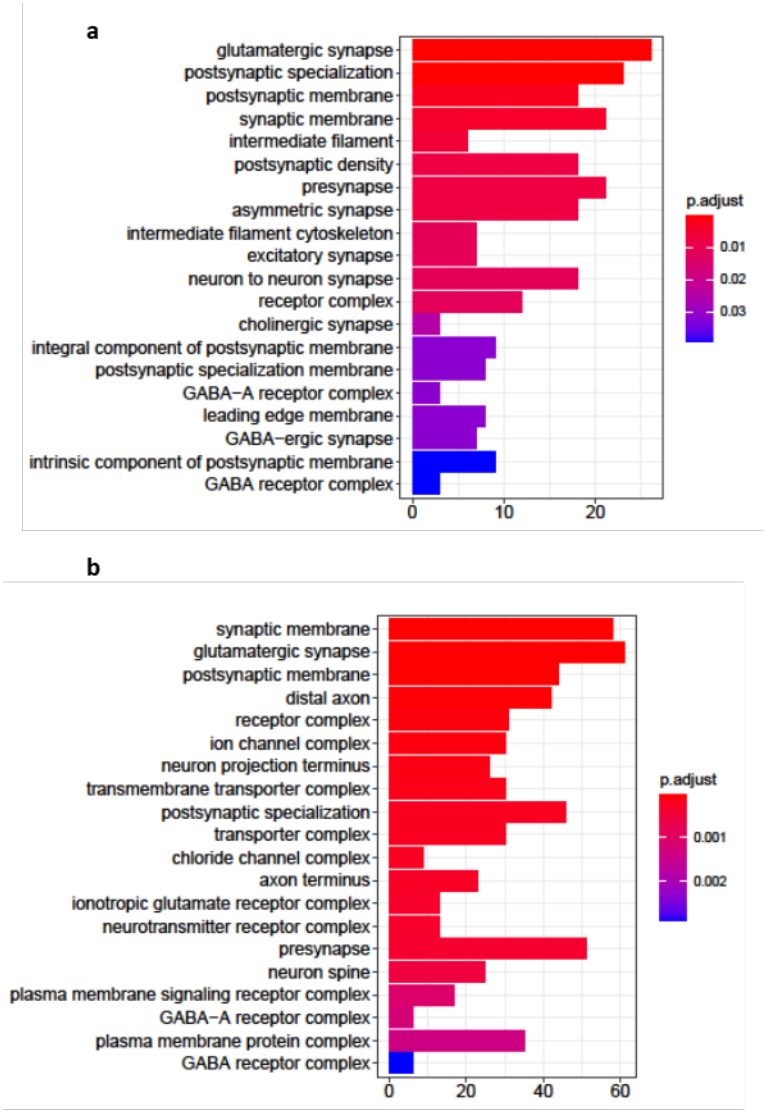


Figure S5. Barplot showing the enrichment analysis of DEGs. The colour gradient indicates the adjusted p-value for the enrichment. Numbers in parentheses indicate the number of identified genes in each category. Bar size corresponds to gene count for each group)/(total gene count for each category). a) Brain-related cellular components where are involved DEGs comparing BE vs STD mice. b) Brain-related cellular components where are involved DEGs comparing BE vs FC mice.

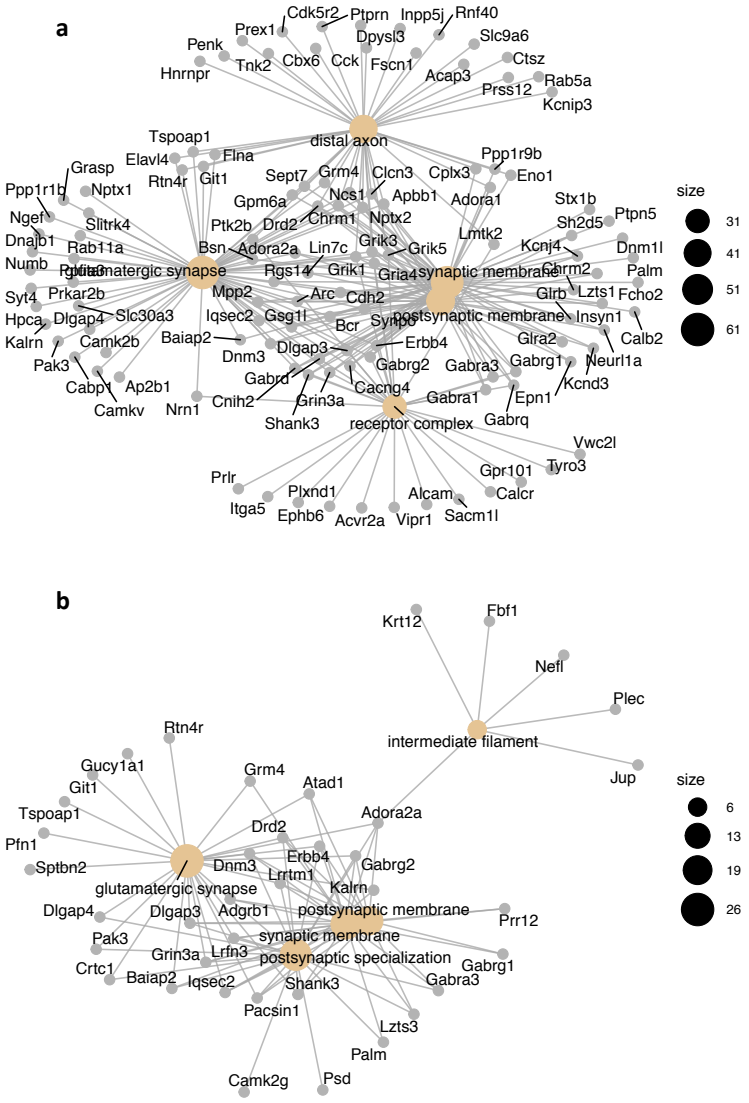


Figure S6. cnetplot showing the enrichment analysis of DEGs. Gene ontology term enrichment cnetplot analysis of DEGs. The brown nodes denote key pathways. The size of the nodes reflects the number of DEGs. Enrichment analysis was performed with a significant FDR < 0.05. a) Brain-related cellular components where are involved DEGs comparing BE vs STD mice. b) Brain-related cellular components where are involved DEGs comparing BE vs FC mice.

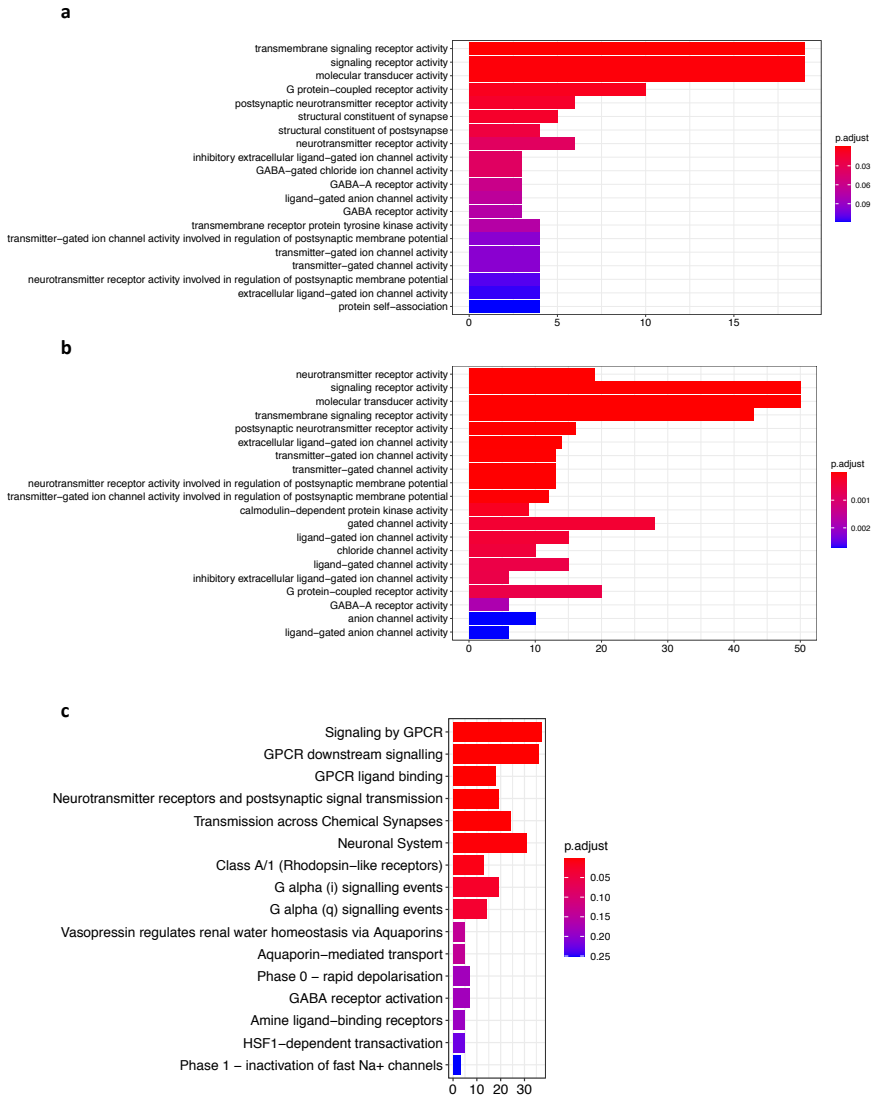


Figure S7. Barplot showing the enrichment analysis of DEGs. The colour gradient indicates the adjusted p-value for the enrichment. Numbers in parentheses indicate the number of identified genes in each category. Bar size corresponds to gene count for each group)/(total gene count for each category). a) Molecular factors where are involved DEGs comparing BE vs STD mice. b) Molecular factors where are involved DEGs comparing BE vs FC mice. c) Reactome analysis

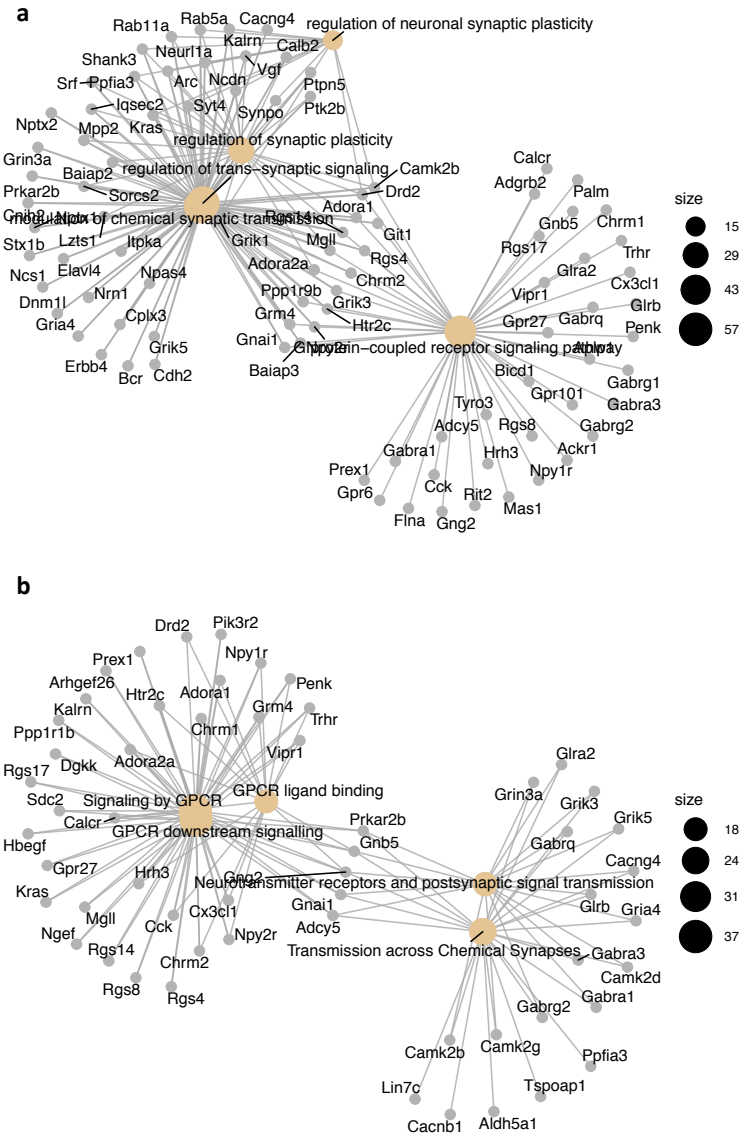


Figure S8. cnetplot showing the enrichment analysis of DEGs. Gene ontology term enrichment cnetplot analysis of DEGs. The brown nodes denote key pathways. The size of the nodes reflects the number of DEGs. Enrichment analysis was performed with a significant FDR < 0.05. a) Molecular factors where are involved DEGs comparing BE vs STD mice. b) Molecular factors where are involved DEGs comparing BE vs FC mice.

T**able S3. Irwing test.** Behavioural parameters measured to detect possible side effects.

tremor	0-2-4-6-8
straub (Straub tail: Tail vertically)	
sedation	
excitation	
abnormal gait (rolling, tip-toe)	0-2-4-6-8
jumping	
loss of balance	
motor incoordination	
writhes (retorcerse dolor)	
piloerection	0-2-4-6-8
stereotypies (sniffing, chewing, head movements)	
head twitches	0-2-4-6-8
scratching	
modified respiration	
fear	
aggressivenes	
reactivity to touch	0-2-4-6-8
ptosis (desprendimiento parpado)	
exophtalmos (Protusion ocular)	
loss of traction	
diarrhoea	
salivation	0-2-4-6-8
lacrimation	

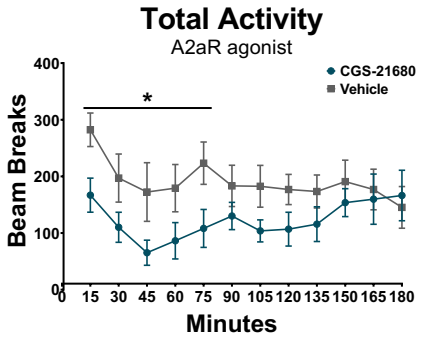


Figure S9. Locomotion test followin CGS 21680 1ng/side injection. Total activity in beam breaks for 3 hours (repeated measures ANOVA test drug*time *p<0.05. mean ± SEM)

Table S3. Pharmacological validation. (Fig. 26, S3)

Figure number	Statistical analysis	Factor name	Statistic value	P-value
Fig. b-d		**2-AG inh. O-7460**		
		Vehicle		
		Time_15	*K-S*= 0.210	n.s
		Time_30	*K-S*= 0.223	n.s
		Time_45	*K-S*= 0.128	n.s
		Time_60	*K-S*= 0.245	n.s
		Time_75	*K-S*= 0.203	n.s
		Time_90	*K-S*= 0.161	n.s
		Time_105	*K-S*= 0.182	n.s
		Time_120	*K-S*= 0.264	P<0.05
		Time_135	*K-S*= 0.262	n.s
		Time_150	*K-S*= 0.153	n.s
		Time_165	*K-S*= 0.255	n.s
		Time_180	*K-S*= 0.209	n.s
		O-7460		
		Time_15	*K-S*= 0.141	n.s
		Time_30	*K-S*= 0.154	n.s
		Time_45	*K-S*= 0.136	n.s
		Time_60	*K-S*= 0.160	n.s
		Time_75	*K-S*= 0.196	n.s
	Kolmogorov-	Time_90	*K-S*= 0.155	n.s
	Smirnov	Time_105	*K-S*= 0.142	n.s
		Time_120	*K-S*= 0.189	n.s
		Time_135	*K-S*= 0.144	n.s
		Time_150	*K-S*= 0.235	n.s
		Time_165	*K-S*= 0.196	n.s
		Time_180	*K-S*= 0.197	n.s
		Cholecystokinin ag. CCK8-S		
		Vehicle		
		Time_15	*K-S*= 0.169	n.s
		Time_30	*K-S*= 0.240	n.s
		Time_45	*K-S*= 0.268	n.s
		Time_60	*K-S*= 0.262	n.s
		Time_75	*K-S*= 0.247	n.s
		Time_90	*K-S*= 0.210	n.s
		Time_105	*K-S*= 0.172	n.s
		Time_120	*K-S*= 0.195	n.s
		Time_135	*K-S*= 0.214	n.s
		Time_150	*K-S*= 0.189	n.s
		Time_165	*K-S*=0.223	n.s
		Time_180	*K-S*= 0.196	n.s

		CCK8-S		
		Time_15	*K-S*= 0.294	n.s
		Time_30	*K-S*= 0.238	n.s
		Time_45	*K-S*= 0.247	n.s
		Time_60	*K-S*= 0.217	n.s
		Time_75	*K-S*= 0.272	n.s
		Time_90	*K-S*= 0.184	n.s
		Time_105	*K-S*= 0.349	p<0.05
		Time_120	*K-S*= 0.233	n.s
		Time_135	*K-S*= 0.362	p<0.05
		Time_150	*K-S*= 0.191	n.s
		Time_165	*K-S*= 0.227	n.s
		Time_180	*K-S*= 0.267	n.s
		A2a R ag. CGS-21680		
		Vehicle		n.s
		Time_15	*K-S*= 0.240	n.s
		Time_30	*K-S*= 0.213	n.s
		Time_45	*K-S*= 0.223	n.s
		Time_60	*K-S*= 0.275	n.s
		Time_75	*K-S*= 0.228	n.s
		Time_90	*K-S*= 0.278	n.s
		Time_105	*K-S*= 0.250	n.s
		Time_120	*K-S*= 0.230	n.s
		Time_135	*K-S*= 0.176	n.s
		Time_150	*K-S*= 0.227	n.s
		Time_165	*K-S*= 0.233	n.s
		Time_180	*K-S*= 0.257	
		CGS-21680		
		Time_15	*K-S*= 0.294	n.s
		Time_30	*K-S*= 0.238	n.s
		Time_45	*K-S*= 0.247	n.s
		Time_60	*K-S*= 0.217	n.s
		Time_75	*K-S*= 0.272	n.s
		Time_90	*K-S*= 0.184	n.s
		Time_105	*K-S*= 0.349	p<0.05
		Time_120	*K-S*= 0.233	n.s
		Time_135	*K-S*= 0.362	p<0.05
		Time_150	*K-S*= 0.191	n.s
		Time_165	*K-S*= 0.227	n.s
		Time_180	*K-S*= 0.267	n.s
		Total locomotion activity		
	Repeated	2-AG inh. O-7460	*F*(11, 198)=0.810	
	measure	Group x time		n.s
	ANOVA		*F*(11, 110)=1.500	
		Cholecystokinin ag. CCK8-S		

		Group x time		n.s
			F(11,110)=3.155	
		CGS-21680		
		Group x time		p<0.05
Fig. e-g		**2-AG inh. O-7460**		
		Vehicle	*K-S*=0.209	n.s
		O-7460	*K-S*=0.158	n.s
		Cholecystokinin ag. CCK8-S		
	Kolmogorov-Smirnov	Vehicle	*K-S*=0.156	n.s
		CCK8-S	*K-S*=0.264	n.s
		A2a R ag. CGS-21680		
		Vehicle	*K-S*=0.156	n.s
		CGS-21680	*K-S*=0.175	n.s
	Levene's test	2-AG inh. O-7460	F=0.583	n.s
		Cholecystokinin ag. CCK8-S	F=0.001	n.s
		A2a R ag. CGS-21680	F=1.926	n.s
	t-test (equal variances assumed)	**2-AG inh. O-7460**		
		Rearing	t=1.199	n.s
		Cholecystokinin ag. CCK8-S		
		Rearing	t=-1.936	n.s
		A2a R ag. CGS-21680		
		Rearing	t=2.125	n.s
Fig. h-j		**2-AG inh. O-7460**		
		Vehicle	K-S=2.33	n.s
		O-7460	K-S=2.94	p<0.05
		Cholecystokinin ag. CCK8-S		
	Kolmogorov-Smirnov	Vehicle		n.s
		CCK8-S	K-S=0.381	n.s
		A2a R ag. CGS-21680	K-S=0.181	
		Vehicle		n.s
		CGS-21680	K-S=0.138	n.s
			K-S=0.243	
	Levene's test	2-AG inh. O-7460		
		Cholecystokinin ag. CCK8-S	F=0.271	n.s
		A2a R ag. CGS-21680	F=2.703	n.s
	t-test (equal variances assumed)	**Cholecystokinin ag. CCK8-S**		
		%time in open arms	t=0,757	n.s
		A2a R ag. CGS-21680		
		%time in open arms	t=3.039	n.s
	U Mann-Whitney	**2-AG inh. O-7460**		
		%time in open arms	U=27.00	n.s

Table S4. Pharmacological validation. (Fig. 27, S4)				
Figure number	**Statistical analysis**	**Factor name**	**Statistic value**	**P-value**
Fig. c, f, i	Kolmogorov-Smirnov	**Cholecystokinin ag. CCK8-S**		
		STD food group		
		Vehicle_STD food 1.5h	*K-S*= 0.310	P<0.05
		Low_Dose_STD food 1.5h	*K-S*= 0.277	n.s
		High_Dose_STD food 1.5h	*K-S*= 0.190	n.s
		Vehicle_STD food 3h	*K-S*= 0.286	n.s
		Low_Dose_STD food 3h	*K-S*= 0.195	n.s
		High_Dose_STD food 3h	*K-S*= 0.256	n.s
		Vehicle_STD food 24h	*K-S*= 0.270	n.s
		Low_Dose_STD food 24h	*K-S*= 0.231	n.s
		High_Dose_STD food 24h	*K-S*= 0.193	n.s
		Binge-eating group		
		Vehicle_STD food 1.5h	*K-S*= 0.280	n.s
		Low_Dose_STD food 1.5h	*K-S*= 0.327	p<0.05
		High_Dose_STD food 1.5h	*K-S*= 0.415	p<0.001
		Vehicle_CD 1.5h	*K-S*= 0.237	n.s
		Low_Dose_CD food 1.5h	*K-S*= 0.276	n.s
		High_Dose_CD 1.5h	*K-S*= 0.301	p<0.05
		Vehicle_STD food 3h	*K-S*= 0.198	n.s
		Low_Dose_STD food 3h	*K-S*=0.159	n.s
		High_Dose_STD food 3h	*K-S*= 0.205	n.s
		Vehicle_CD 3h	*K-S*= 0.200	n.s
		Low_Dose_CD food 3h	*K-S*= 0.199	n.s
		High_Dose_CD 3h	*K-S*= 0.223	n.s
		Vehicle_STD food 24h	*K-S*= 0.273	n.s
		Low_Dose_STD food 24h	*K-S*= 0.181	n.s
		High_Dose_STD food 24h	*K-S*= 0.214	n.s
		Vehicle_CD 24h	*K-S*= 0.246	n.s
		Low_Dose_CD food 24h	*K-S*= 0.180	n.s
		High_Dose_CD 24h	*K-S*= 0.263	n.s
	Friedman Test (Non-parametric test)	STD food group 1.5h	$\chi 2$ (2)= 2.138	n.s
		Binge-eating group		
		STD food intake 1.5h	$\chi 2$ (2)= 0.875	n.s
		CD intake 1.5h	$\chi 2$ (2)= 2.385	n.s
	Repeated measures ANOVA	STD food group 3h	*F*(2,14)= 1.273	n.s
		Binge-eating group		
		STD food intake 3h	*F*(2,14)= 0.058	n.s
		CD intake 3h	*F*(2,14)= 0.394	n.s
		STD food group 24h	*F*(2,14)= 0.721	n.s
		Binge-eating group		
		STD food intake 24h	*F*(2,14)= 0.007	n.s
		CD intake 24h	*F*(2,14)= 1.242	n.s

Fig. d, g, j	Kolmogorov-Smirnov	**A2aR ag. CGS 21680**		
		STD food group		
		Vehicle_STD food 1.5h	*K-S*= 0.187	n.s
		Low_Dose_STD food 1.5h	*K-S*= 0,197	n.s
		High_Dose_STD food 1.5h	*K-S*= 0.129	n.s
		Vehicle_STD food 3h	*K-S*= 0.163	n.s
		Low_Dose_STD food 3h	*K-S*= 0.326	p<0.01
		High_Dose_STD food 3h	*K-S*= 0.138	n.s
		Vehicle_STD food 24h	*K-S*= 0.274	p<0.05
		Low_Dose_STD food 24h	*K-S*= 0.238	n.s
		High_Dose_STD food 24h	*K-S*= 0.188	n.s
		Binge-eating group		
		Vehicle_STD food 1.5h	*K-S*= 0.297	p<0.01
		Low_Dose_STD food 1.5h	*K-S*= 0.256	p<0.01
		High_Dose_STD food 1.5h	*K-S*= 0.289	p<0.01
		Vehicle_CD 1.5h	*K-S*= 0.166	n.s
		Low_Dose_CD food 1.5h	*K-S*= 0.146	n.s
		High_Dose_CD 1.5h	*K-S*= 0.102	n.s
		Vehicle_STD food 3h	*K-S*= 0.201	n.s
		Low_Dose_STD food 3h	*K-S*= 0.222	p<0.05
		High_Dose_STD food 3h	*K-S*= 0.283	p<0.01
		Vehicle_CD 3h	*K-S*= 0.129	n.s
		Low_Dose_CD food 3h	*K-S*= 0.158	n.s
		High_Dose_CD 3h	*K-S*= 0.138	n.s
		Vehicle_STD food 24h	*K-S*= 0.152	n.s
		Low_Dose_STD food 24h	*K-S*= 0.159	n.s
		High_Dose_STD food 24h	*K-S*= 0.128	n.s
		Vehicle_CD 24h	*K-S*= 0.140	n.s
		Low_Dose_CD food 24h	*K-S*= 0.190	n.s
		High_Dose_CD 24h	*K-S*= 0.145	n.s
	Friedman Test (Non-parametric test)	STD food group 1.5h	$\chi 2$ (2)= 3.211	n.s
		Binge-eating group		
		STD food intake 1.5h	$\chi 2$ (2)= 0.255	n.s
		CD intake 1.5h	$\chi 2$ (2)= 11.828	p<0.01
		STD food group 3h	$\chi 2$ (2)= 11.697	p<0.01
		Binge-eating group		
		STD food intake 3h	$\chi 2$ (2)= 0.255	n.s
		CD intake 3h	$\chi 2$ (2)= 14.400	p<0.01
		STD food group 24h	$\chi 2$ (2)= 2.400	n.s
		Binge-eating group		
		STD food intake 24h	$\chi 2$ (2)= 4.508	n.s

		CD intake 24h	χ2 (2)= 8.400	p<0.05
	Wilcoxon Posthoc	CD intake 1.5h		
		Veh. *vs* low dose	Z= -1.163	n.s
		Veh. *vs* high dose	Z= -3.078	p<0.01
		Low dose *vs* high dose	Z= -2.501	p<0.05
		STD food group 3h		
		Veh. *vs* low dose	Z= -0.526	n.s
		Veh. *vs* high dose	Z= -2.675	p<0.01
		Low dose *vs* high dose	Z= -2.439	p<0.05
		CD intake 3h		
		Veh. *vs* low dose	Z= -1.223	n.s
		Veh. *vs* high dose	Z= -2.953	p<0.01
		Low dose *vs* high dose	Z= -2.841	p<0.01
Fig. e, h, k	Kolmogorov-Smirnov	**2-AG inh. O-7460**		
		STD food group		
		Vehicle_STD food 1.5h	K-S= 0.170	n.s
		High_Dose_STD food 1.5h	K-S= 0.192	n.s
		Vehicle_STD food 3h	K-S= 0.149	n.s
		High_Dose_STD food 3h	K-S= 0.192	n.s
		Vehicle_STD food 24h	K-S= 0.306	p<0.01
		High_Dose_STD food 24h	K-S=0.250	p<0.05
		Binge-eating group		
		Vehicle_STD food 1.5h	K-S= 0.401	p<0.001
		High_Dose_STD food 1.5h	K-S= 0.2.03	n.s
		Vehicle_CD 1.5h	K-S= 0.157	n.s
		High_Dose_CD 1.5h	K-S= 0.275	p<0.05
		Vehicle_STD food 3h	K-S= 0.312	n.s
		High_Dose_STD food 3h	K-S= 0.161	n.s
		Vehicle_CD 3h	K-S= 0.157	n.s
		High_Dose_CD 3h	K-S= 0.153	n.s
		Vehicle_STD food 24h	K-S= 0.175	n.s
		High_Dose_STD food 24h	K-S= 0.178	n.s
		Vehicle_CD 24h	K-S= 0.131	n.s
		High_Dose_CD 24h	K-S= 0.173	n.s
	Wilcoxon signed-rank test	STD food group 1.5h	*t*=1.353	n.s
		Binge-eating group		
		STD food intake 1.5h	*t*= 2.264	p<0.05
		CD intake 1.5h	*t*= -0.843	n.s
		STD food group 3h	*t*= -1.740	n.s
		Binge-eating group		
		STD food intake 3h	*t*= 1.121	n.s
		CD intake 3h	*t*= -2.706	p<0.01
		STD food group 24h	*t*= 0.591	n.s

		Binge-eating group		
		STD food intake 24h	t= 2.269	p<0.05
		CD intake 24h	t= -2.293	p<0.05

Table S6. Top 50 up- and down-regulated genes in nucleus accumbens D2(+) neurons of D2:Cre-RiboTag male mice comparing Binge eating vs standard groups

DE Binge vs Standard

UP				DOWN			
Gene	log2FC	lfcSE	padj	Gene	log2FC	lfcSE	padj
Meg3	0,537	0,088	1,34E-05	*Fstl5*	-0,488	0,096	2,92E-04
Cck	0,526	0,098	1,59E-04	*Slc35d3*	-0,481	0,092	2,56E-04
Rtn4r	0,448	0,098	1,65E-03	*Slc17a6*	-0,407	0,097	3,97E-03
Ttll3	0,447	0,097	1,65E-03	*Gabrg1*	-0,405	0,087	1,30E-03
Slc30a3	0,441	0,094	1,17E-03	*Krt12*	-0,405	0,096	3,97E-03
Clspn	0,401	0,098	5,13E-03	*Prkra*	-0,402	0,093	3,19E-03
Npas4	0,388	0,095	5,24E-03	*Ndn*	-0,397	0,075	1,93E-04
Ccdc88c	0,379	0,089	3,27E-03	*Ddc*	-0,375	0,093	5,71E-03
Ubald1	0,376	0,068	1,43E-04	*Npy1r*	-0,369	0,092	5,86E-03
Pou3f1	0,374	0,083	1,90E-03	*Twistnb*	-0,366	0,093	6,84E-03
Nfix	0,369	0,095	7,73E-03	*AW551984*	-0,365	0,088	4,47E-03
Hist1h2be	0,364	0,097	9,89E-03	*Ankrd29*	-0,360	0,098	1,10E-02
Prss12	0,364	0,093	7,20E-03	*Gabrq*	-0,356	0,098	1,18E-02
Coch	0,359	0,097	1,04E-02	*Igsf1*	-0,355	0,098	1,22E-02
Cdk2ap2	0,352	0,094	9,85E-03	*Vbp1*	-0,354	0,083	3,26E-03
AI480526	0,351	0,096	1,17E-02	*Erbb4*	-0,351	0,092	8,91E-03
Plxdc1	0,348	0,086	5,30E-03	*Il13ra1*	-0,351	0,097	1,26E-02
Lrfn3	0,347	0,068	2,92E-04	*Optn*	-0,351	0,098	1,36E-02
Hrh3	0,346	0,070	5,30E-04	*Rasgrf2*	-0,350	0,096	1,24E-02
Miip	0,346	0,097	1,39E-02	*Glmn*	-0,348	0,092	9,28E-03
Suv420h2	0,345	0,095	1,26E-02	*Dpp10*	-0,348	0,068	2,78E-04
Snrpc	0,340	0,077	2,93E-03	*Ttbk2*	-0,347	0,086	5,76E-03
Mocs1	0,337	0,093	1,26E-02	*Sulf1*	-0,342	0,088	7,39E-03
Zfpm1	0,336	0,086	7,20E-03	*Sema3c*	-0,338	0,097	1,74E-02
Mov10	0,335	0,093	1,26E-02	*Itga9*	-0,336	0,091	1,07E-02
Rasl10a	0,333	0,087	8,36E-03	*Slc35g2*	-0,333	0,092	1,26E-02
Mbd6	0,329	0,076	3,26E-03	*Grin3a*	-0,332	0,087	8,36E-03
Timm13	0,328	0,068	7,81E-04	*C77370*	-0,332	0,088	9,19E-03
E130012A19Rik	0,326	0,087	9,40E-03	*Cd83*	-0,332	0,090	1,11E-02
Dlgap3	0,325	0,074	2,98E-03	*Lhfpl3*	-0,332	0,079	3,81E-03
Col6a1	0,323	0,063	2,78E-04	*Fam19a2*	-0,331	0,084	7,20E-03
Gpr6	0,322	0,066	6,23E-04	*Lrch2*	-0,330	0,091	1,18E-02
Gpr27	0,320	0,083	8,43E-03	*Rnf152*	-0,330	0,096	1,90E-02
Nabp2	0,316	0,075	3,97E-03	*Xpo1*	-0,329	0,077	3,59E-03
Clasrp	0,314	0,083	8,97E-03	*Gpr101*	-0,328	0,087	9,19E-03
Gm3764	0,314	0,092	1,91E-02	*Klhl4*	-0,328	0,098	2,23E-02
Nrn1	0,313	0,098	2,92E-02	*Pcdh19*	-0,326	0,085	8,30E-03
Ppp1r3d	0,311	0,098	2,98E-02	*Kitl*	-0,324	0,076	3,26E-03
Exosc5	0,310	0,082	9,13E-03	*Fgf10*	-0,320	0,095	2,10E-02
Gltscr1	0,308	0,086	1,39E-02	*Trhde*	-0,319	0,094	1,94E-02
Rgs6	0,308	0,096	2,86E-02	*A830036E02Rik*	-0,319	0,089	1,28E-02
Slit3	0,307	0,070	2,93E-03	*Asah2*	-0,319	0,091	1,55E-02
Syndig1l	0,307	0,075	5,09E-03	*Tab3*	-0,319	0,077	4,85E-03
Grin2c	0,306	0,098	3,26E-02	*Pkia*	-0,317	0,075	3,81E-03
Rfx1	0,306	0,075	5,30E-03	*Cryz*	-0,314	0,081	7,87E-03
Ephb6	0,305	0,076	5,81E-03	*Sema3a*	-0,310	0,086	1,30E-02
Pitpnm3	0,305	0,080	8,75E-03	*Nefl*	-0,310	0,072	3,19E-03
Spen	0,301	0,068	2,54E-03	*Nts*	-0,309	0,098	3,15E-02
Mesdc1	0,300	0,078	7,87E-03	*Phyh*	-0,309	0,085	1,23E-02
Nat6	0,299	0,084	1,45E-02	*Unc5d*	-0,308	0,083	1,03E-02

Genes are ranked by logFC. adj.p.val, FDR-adjusted p value; logFC, log2 fold-change

Table S7. Top 50 up- and down-regulated genes in nucleus accumbens D2(+) neurons of D2:Cre-RiboTag male mice comparing Binge eating vs free-choice groups

DE Binge vs FC							
UP				DOWN			
Gene	log2FC	lfcSE	padj	Gene	log2FC	lfcSE	padj
Cplx3	0,582	0,098	2,92E-06	*Gabrg1*	-0,589	0,086	3,90E-08
Nptx2	0,535	0,079	3,90E-08	*Gabrq*	-0,543	0,098	1,69E-05
Cck	0,505	0,098	7,77E-05	*AW551984*	-0,531	0,088	1,98E-06
Itga5	0,496	0,091	2,39E-05	*Sema3c*	-0,523	0,097	3,56E-05
Slc30a3	0,483	0,094	8,45E-05	*Tspan2*	-0,518	0,084	1,33E-06
Mas1	0,466	0,094	1,78E-04	*Edil3*	-0,507	0,088	6,69E-06
Prss12	0,460	0,093	1,72E-04	*Lhfpl3*	-0,504	0,078	2,75E-07
Nxph3	0,454	0,095	2,79E-04	*Gpr101*	-0,488	0,086	1,28E-05
Ccnd2	0,449	0,088	8,61E-05	*AI504432*	-0,482	0,094	8,44E-05
Npas4	0,449	0,095	3,26E-04	*Marchf7*	-0,468	0,077	1,75E-06
Nr4a2	0,446	0,095	3,82E-04	*Slc17a6*	-0,467	0,097	2,55E-04
Tbr1	0,417	0,097	1,14E-03	*Chrm2*	-0,460	0,096	2,76E-04
Gm11549	0,416	0,085	2,19E-04	*Lrrcc1*	-0,458	0,090	9,49E-05
Clspn	0,411	0,098	1,43E-03	*Pkia*	-0,454	0,075	1,98E-06
Pdzrn3	0,411	0,098	1,43E-03	*Zfp758*	-0,453	0,098	4,44E-04
Ctgf	0,406	0,089	5,43E-04	*Vstm2a*	-0,447	0,080	1,69E-05
Dusp14	0,385	0,078	1,70E-04	*Gm17066*	-0,434	0,084	8,31E-05
Rtn4r	0,381	0,098	3,29E-03	*Erbb4*	-0,433	0,092	3,60E-04
Fmnl1	0,376	0,084	6,36E-04	*Lhx6*	-0,433	0,088	1,88E-04
Kremen1	0,370	0,079	3,60E-04	*Vwc2l*	-0,430	0,094	5,25E-04
Nrn1	0,367	0,098	4,83E-03	*Zxdb*	-0,429	0,098	8,37E-04
Vipr1	0,364	0,075	2,27E-04	*Asah2*	-0,428	0,090	3,01E-04
Fam53b	0,361	0,067	3,78E-05	*Vma21*	-0,425	0,087	2,19E-04
Dmkn	0,355	0,093	4,00E-03	*Pcdh11x*	-0,424	0,096	7,77E-04
Cd4	0,353	0,080	7,52E-04	*Xpo1*	-0,423	0,077	2,39E-05
Bsn	0,353	0,051	3,90E-08	*Fstl5*	-0,421	0,096	8,37E-04
Sorcs2	0,352	0,075	3,82E-04	*Nap1l5*	-0,420	0,065	2,75E-07
Ccnd1	0,350	0,096	6,09E-03	*Tmx3*	-0,414	0,082	1,05E-04
Dok3	0,350	0,098	7,39E-03	*Mgat4c*	-0,413	0,092	6,36E-04
Gdpd5	0,343	0,072	2,79E-04	*Megf10*	-0,413	0,098	1,35E-03
Rasd2	0,343	0,067	9,34E-05	*Lrch2*	-0,412	0,090	5,31E-04
Ephb6	0,339	0,076	6,74E-04	*Ankrd29*	-0,410	0,098	1,39E-03
Hbegf	0,337	0,088	3,70E-03	*Klhl4*	-0,410	0,098	1,43E-03
Dedd2	0,336	0,077	9,23E-04	*Ap1s2*	-0,406	0,073	1,69E-05
Flna	0,335	0,079	1,32E-03	*Fcho2*	-0,405	0,094	1,02E-03
Arc	0,333	0,079	1,35E-03	*Rnf152*	-0,402	0,096	1,43E-03
Wipf3	0,332	0,058	1,06E-05	*Chpt1*	-0,400	0,098	1,95E-03
E130012A19Rik	0,330	0,086	3,84E-03	*Cd164*	-0,399	0,089	6,43E-04
Gpr27	0,330	0,083	2,75E-03	*Hecw1*	-0,399	0,085	3,45E-04
Plch2	0,328	0,071	4,61E-04	*Nhs*	-0,399	0,094	1,23E-03
Syndig1l	0,325	0,075	1,00E-03	*Acvr2a*	-0,398	0,081	1,93E-04
Kctd17	0,317	0,063	1,41E-04	*Plcxd2*	-0,397	0,091	9,59E-04
Gpr6	0,317	0,066	2,79E-04	*Calcr*	-0,396	0,088	6,43E-04
Rbm3	0,316	0,071	7,53E-04	*Kbtbd7*	-0,393	0,086	5,43E-04
Slit3	0,315	0,070	5,95E-04	*Cd83*	-0,390	0,090	9,49E-04
Lrrc32	0,315	0,097	1,63E-02	*Lingo2*	-0,389	0,096	2,27E-03
Abcc12	0,313	0,098	1,82E-02	*Slitrk4*	-0,387	0,085	5,43E-04
P4ha1	0,311	0,064	2,61E-04	*Pcdh19*	-0,385	0,085	5,43E-04
Zbtb40	0,310	0,075	1,71E-03	*Vbp1*	-0,384	0,082	3,82E-04
Grasp	0,309	0,068	5,43E-04	*Elavl2*	-0,384	0,087	8,08E-04

Genes are ranked by logFC. adj.p.val, FDR-adjusted p value; logFC, log2 fold-change

Study 2

A Gallego-Román[1], R Vindas[3], R. Maldonado[1,2*], E. Martín-García[1*], M. Martín-Sánchez[1#*].

[1]. University Pompeu Fabra, Barcelona, Spain.

[2]. IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain.

[3]. Universidad de Costa Rica, INSA.

[#] Current address: Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain.

[*] Equally contributed.

Under preparation

Adenosine A_{2a} receptor over-expression into the nucleus accumbens to the ventral pallidum pathway decreases motivation for chocolate in binge-like eating mice

Selective upregulation of A_{2A}Rs in the indirect pathway of the NAc

To investigate the role of A_{2A}R in the indirect ventral pathway in a BE phenotype, we induced a specific overexpression of A_{2A}R in NAc core-VP projections using a dual viral vector approach with a Cre-dependent AAV-A2aR-mCherry (n = 11 and 13) and AAV-CTRL-mCherry (n = 11 and 15) injected into NAc, together with an AAV-retrograde-Cre-BFP injected into the VP (**Figure 28a**). The location of AAV-injection sites was verified by immunofluorescence against Cre recombinase and mCherry detection showing that viral-mediated A_{2A}R expression was specific to the indirect projection pathway since mCherry were observed exclusively in the projection target of NAc and the Cre-recombinase enzyme immunoreactive in the VP (**Figure 28b**). In addition, we demonstrated that this viral strategy resulted in a more than twenty-fold change increase in A_{2A}R mRNA by quantitative real-time PCR analysis (qPCR) (**Figure 28c**).

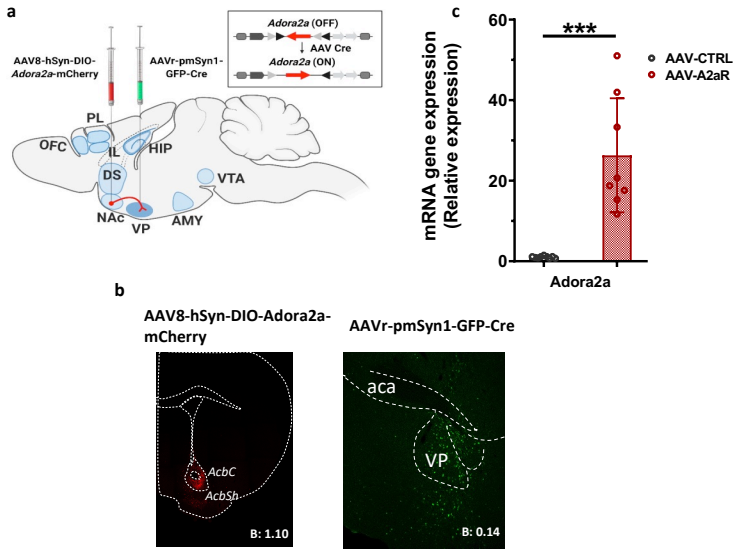


Figure 28. A_{2a}R over-expression by viral vector-approach. a) Scheme of a combinatorial viral strategy for selective A_{2a}R over-expression in NAc core-VP projection neurons. **b)** Representative histological validation images showing Cre-induced A_{2a} protein receptor by mCherry reporter gene at NAc core injection site (left) and Cre-recombinase enzyme at VP injection site (right). Higher magnification image shows Cre-recombinase immunolabeling in the VP. Scale =250 µm. **c)** qPCR validation results showing the relative mRNA expression of A_{2a}R in NAc. (t-test. ***p<0.001. AAV-CTRL n=8; AAV-A2a n=8. Individual values with the median and the interquartile range).

A_{2A}R overexpression in NAc core-VP projections reduces energy intake escalation from palatable food during binge-like eating episodes

Then, we investigated the effect of A_{2A}R overexpression in NAc indirect pathway in palatable food intake motivation during binge-eating episodes. 50 WT C57BL/6J male mice were divided into four experimental groups according to the injection and diet: AAV-CTRL-STD; AAV-A2aR-STD; AAV-CTRL-BE; AAV-A2aR-BE. All animals underwent the experimental protocol depicted in Figure 29.

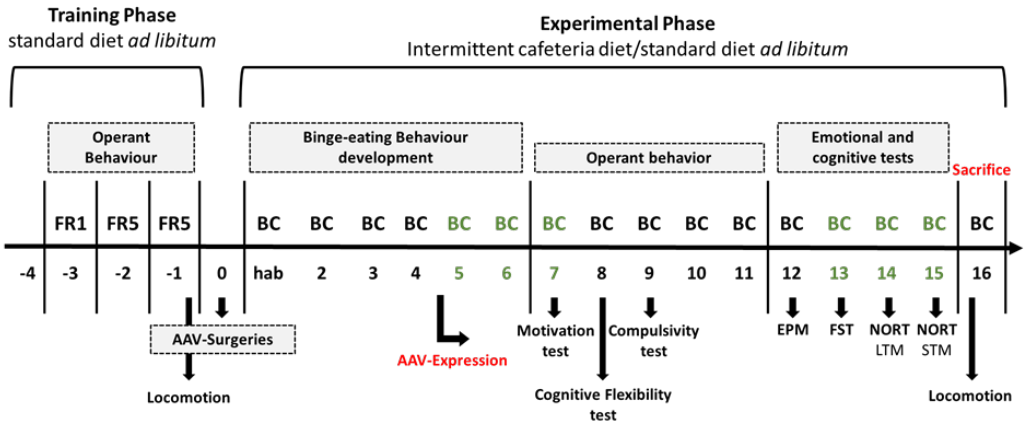


Figure 29. Timeline of the experimental sequence of a combinatorial procedure of two behavioural protocols: the BE eating behaviour model and self-administration behavioural model. Mice were trained to acquire the operant conditioning maintained by chocolate-flavoured pellets (training phase) followed by the surgery for injecting Cre-dependent AAVs carrying the A2aR (beginning of experimental phase). Before the surgery, locomotion and the basal feeding pattern were recorded. The expression of the AAV was allowed for four weeks. Following this, the food intake was compared to the basal feeding pattern established before AAV surgeries (highlighted in green the BCs used for the study). The BCs recorded during operant self-administration protocol were discarded. The operant self-administration protocol was performed from the 7th week to the 11th week. Later, emotional tests (anxiety- and depressive-like behaviour) and cognitive tests (long- and short-term memory) were performed before sacrifices.

To determine changes in motivation for high reinforcing food (preferred food, cafeteria diet), we calculated the relative difference (RD) of energy intake comparing the kCal/gr animal consumed after surgeries during BCs to the baseline conditions before surgeries for both types of diet, CD and StdF (see “experimental procedure”, study 2 in material and methods section). The body weight and the number of active-lever pressing in the last three training phase sessions were considered the main criteria for animal group distribution. Therefore, once diet groups were established (BE and STD groups), the meal pattern at different time points was determined among pre-surgeries groups to determine homogeneity of

food intake pattern among animal groups before surgery. Pre-surgeries groups did not show significant differences in the total energy intake at different time points (1.5 h, 3 h and 24 h) (STD group: StdF, injection_group $F_{(1, 57)}$= 3.299 n.s; BE group: StdF, injection_group $F_{(1, 78)}$= 0.054 n.s; CD, $F_{(1, 77)}$= 0.390 n.s) (Figure 30a-c). Thus, homogeneous feeding patterns between injection groups were established as basal conditions.

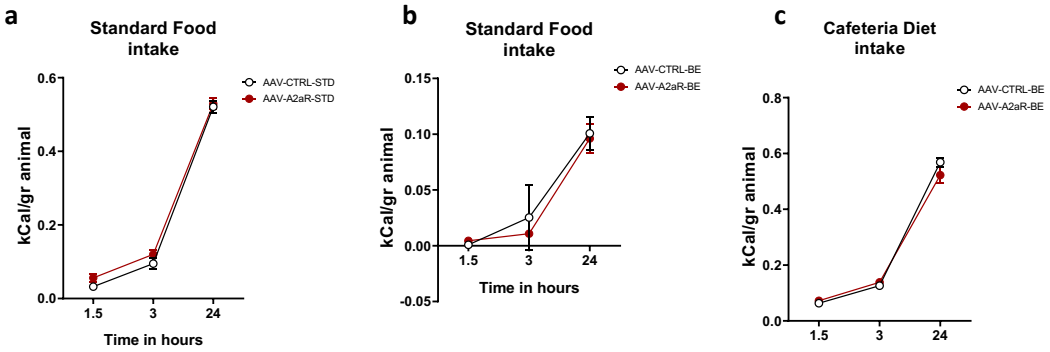


Figure 30. Comparison of absolute food intake values in kCal/gr animal injection groups before surgeries. Data are expressed as mean ± SEM **a)** StdF intake in kCal/gr animal in STD groups at the different time points (1.5 h, 3 h and 24 h). **b)** StdF intake in kCal/gr animal in BE groups at the different time points (1.5 h, 3 h and 24 h). **c)** CD intake in kCal/gr animal in BE groups at the different time points (1.5 h, 3 h and 24 h). Statistical details are included in supplementary table S1.

The progressive escalation in energy intake through BCs was compared to determine differences in food-seeking behaviour among injection groups in each type of diet (**Figure 31**). During the first 1.5 h from the BC onset, mice receiving AAV-A2aR injection significantly differed from those with AAV-CTRL injection in the total energy intake. Mice subjected to intermittent free-choice access to CD consumed significantly less kCal/gr animal relative to the baseline conditions from both types of diet, StdF and CD (StdF: injection_group $F_{(1, 155)}$= 1.966 $p<0.01$; CD injection_group $F_{(1,}$

$_{156)}$= 9.726 p<0.01) (**Figure 31b and c**). On the other hand, during this period, we also found significant differences in the progressive escalation in energy intake in those mice with *ad libitum* access to StdF (injection_group $F_{(1, 114)}$= 5.381 p<0.05) (**Figure 31a**). Following 3 h from CD presentation, no significant differences in the relative consumption of StdF were found for any group (BE and STD) between injection groups (**Figure 31d and e**). Nevertheless, the progressive increase in energy consumption from palatable food was significantly lower in AAV-A2aR-BE mice as compared to AAV-CTRL-BE (injection_group: $F_{(1, 156)}$= 27.658 p<0.001; BC: $F_{(5, 156)}$=2.414 p<0.05) (**Figure 31f**). Data from the 24 h-period revealed no significant changes between groups of injection in any type of diet (STD group: StdF injection_group $F_{(5,114)}$=2.713 n.s; BE group: StdF injection_group $F_{(1,150)}$= 2.362 n.s, CD injection_group $F_{(1,156)}$= 0.307 n.s) (**Figure 31g-i**).

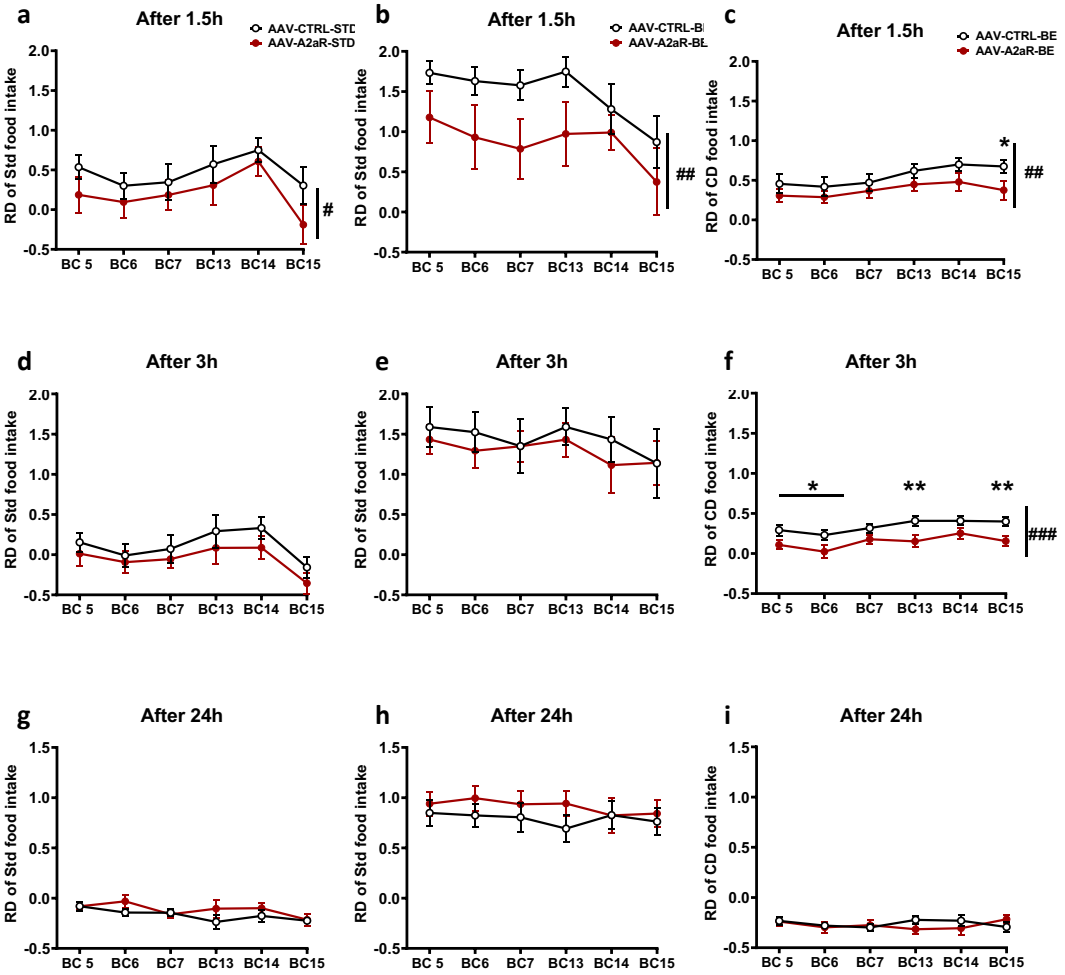


Figure 31. Relative difference of food intake escalation patterns through BCs. Mix model test and Bonferroni's multiple comparison test. Mean ± SEM. a-c) RD of food intake after 1.5 h from BC onset (Injection_group #p<0.05, ##p<0.01). a) RD of StdF intake in the STD group. b) RD of StdF intake in BE group. c) RD of CD intake in BE group (AAV-CTRL vs AAV-A2aR in BC15 *p<0.05). d-f) RD of food intake after 3 h from BC onset. d) RD of StdF intake in STD group. e) RD of StdF intake in BE group. f) RD of CD intake in BE group (Injection_group ##p<0.001; AAV-CTRL vs AAV-A2Ar in each BC *p<0.05, **p<0.01). g-i) RD of food intake after 24 h from BC onset. g) RD of StdF intake in STD group. h) RD of StdF intake in BE group. i) RD of CD intake in BE group. Statistical details are included in supplementary table S1.

In addition, a general comparison between injection groups showed significant differences in the total energy intake, especially in the BE group after 1.5 h and 3 h, but not following 24 h from BC onset (StdF 1.5 h AAV-CTRL vs AAV-A2aR $F_{(1, 75)}$= 4.606 p<0.05; CD 1.5 h AAV-CTRL vs AAV-A2aR $F_{(1, 78)}$=4.178 p<0.05 and 3 h AAV-CTRL vs AAV-A2Ar $F_{(1, 78)}$=5.114 p<0.05) (**Figure 32b and c**). These significant results were not observed in those mice belonging to the STD group with *ad libitum* access to standard food (**Figure 32a**). These results suggested that $A_{2A}R$ upregulation in the indirect ventrostriatal pathway modifies the palatable food intake motivation by reducing the progressive escalation of energy consumption from palatable food during binge-like eating episodes.

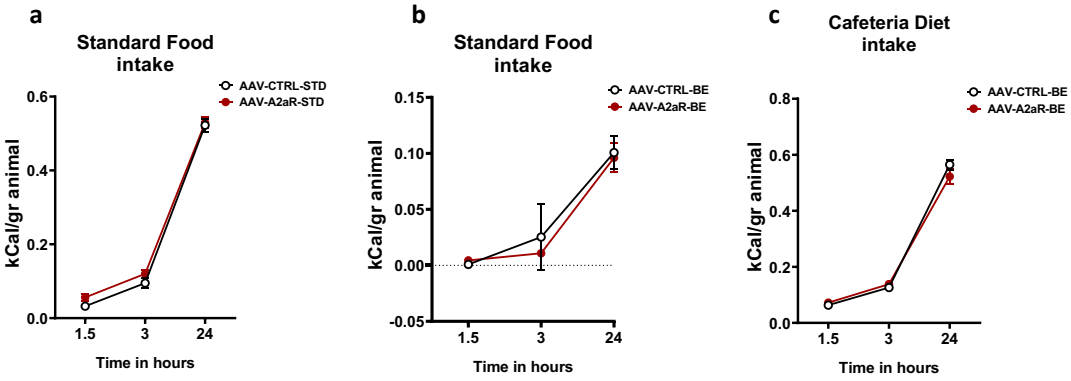


Figure 32. Relative difference (RD) mean of food intake compared between injection groups at different time points. a) RD mean of StdF intake comparison in STD groups at 1.5 h, 3 h and 24 h from BC onset (Mix model test. Mean ± SEM). **b)** RD mean of StdF intake comparison in BE groups at 1.5 h, 3 h and 24 h from BC onset (Mix model test. AAV-CTRL vs AAV-A2aR *p<0.05 Mean ± SEM). **c)** RD means of CD intake comparison in BE groups at 1.5 h, 3 h and 24 h from BC onset (Mix model test. AAV-CTRL vs AAV-A2aR *p<0.05. Mean ± SEM). Statistical details are included in supplementary table S1.

Selective $A_{2A}R$ upregulation reduce motivation for palatable food

Binge-eating disorder and food addiction are highly comorbid, and the differences between both disorders are unclear. Thus, to assess the effect of repetitive binge eating and the $A_{2A}R$ upregulation in the NAc indirect pathway on different hallmarks of addiction, we combined the model of BE behaviour with the operant model of palatable food self-administration paradigm. AAV-CTRL-STD, AAV-CTRL-BE, AAV-A2aR-STD and AAV-A2aR-BE mice were trained under a fixed ratio (FR) 1 schedule of reinforcement during five sessions followed by seven sessions under FR5 to obtain chocolate-flavoured pellets as reinforcers (**Figure 33**). All groups increased the number of reinforcers across sessions during FR1 and FR5 schedule without significant differences and achieved the acquisition criteria (>25% of responses of all FR5 sessions), indicating similar levels of acquisition of the operant conditioning learning at the end of the training phase. No significant changes were observed during the rest of the operant conditioning maintenance by chocolate-flavoured pellets (Training phase: FR1 group*session: $F_{(3, 44)}$= 1.519, n.s; FR5 group*sessions: $F_{(3, 44)}$= 1.540 n.s; Experimental phase: group*sessions $F_{(3, 44)}$= 1.144 n.s).

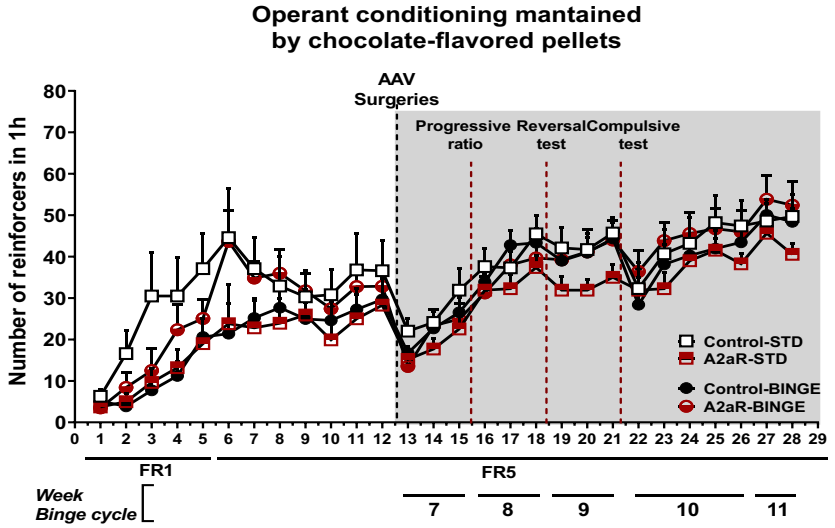


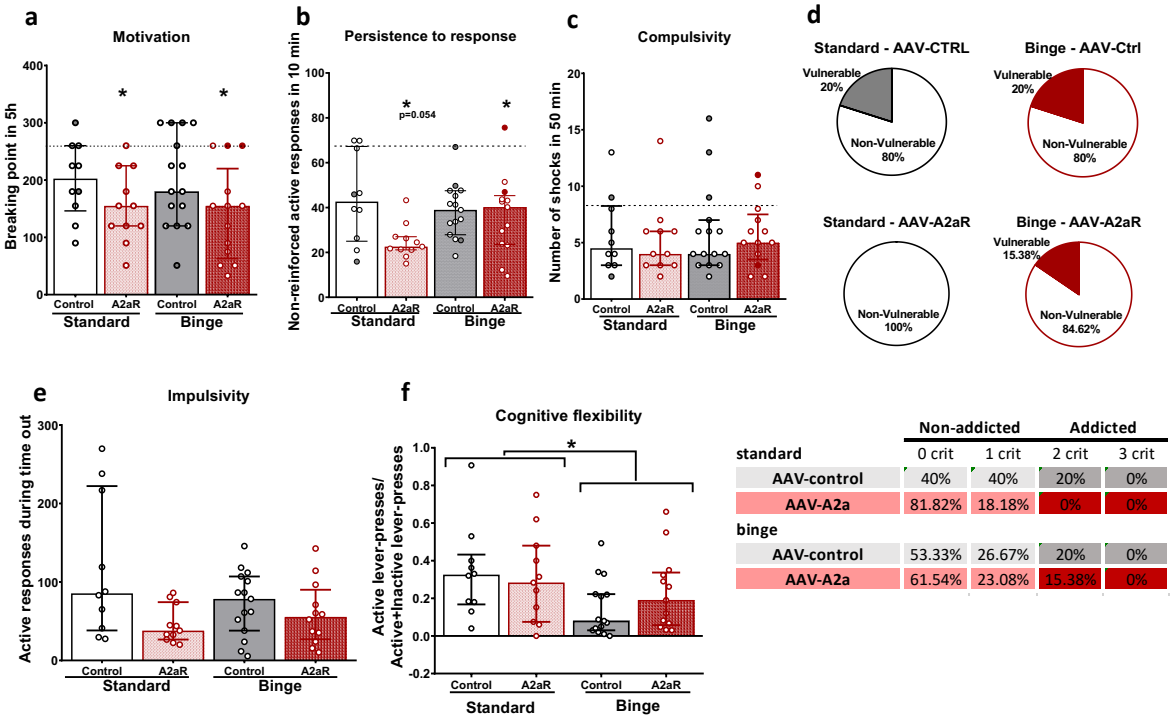
Figure 33. Number of reinforcers during operant conditioning sessions maintained by chocolate-flavoured pellets. Training phase in white and experimental phase from AAV-expression in grey (repeated measures ANOVA, mean ± SEM). Statistical details are included in supplementary table S2.

We used the food addiction-like criteria recently established (Mancino et al., 2015) to evaluate food addiction vulnerability depending on the dietary conditions and determine whether the local overexpression of $A_{2A}R$ in the NAc indirect pathway may influence vulnerability to food addiction. After a training period and stable acquisition of the operant conditioning learning, we assessed the effect of $A_{2A}R$ upregulation on the willingness of mice to work for a palatable food as a reward by a progressive ratio (PR) task. We observed a significant reduction in the breaking point in 5 h of test in the group of mice overexpressing $A_{2A}R$, independently of the diet group, in comparison with those mice with AAV-CTRL injection (injection $F_{(1, 48)}= 5.616$, $p<0.05$) **Figure 34a**). These results revealed that mice overexpressing $A_{2A}R$ were susceptible to ratio requirements in operant schedules but not during low work demand (FR1 and FR5) (**Figure 33**). In addition, significant differences were detected

among injection groups in the persistence to response (injection $F_{(1, 48)}$= 6.052, $p<0.05$). Mice overexpressing $A_{2A}R$ in the NAc indirect pathway and fed with *ad libitum* access to standard food tended to decrease the number of non-reinforced active responses during the pellet-free period (10 min) in comparison to the control-injection group (AAV-CTRL-STD) and both injection groups with intermittent free-choice access to CD (AAV-CTRL-BE and AAV-A2aR-BE) (**Figure 34b**). We did not observe any change in the criterion of compulsivity evaluated by the number of active responses associated with a foot-shock delivery (**Figure 34d**).

Two additional phenotypic traits considered as factors of vulnerability to addiction, impulsivity and cognitive flexibility, were also evaluated. No changes were observed between injection groups and diet regarding impulsivity (**Figure 34f**). Nevertheless, we observed significant differences between groups of diet in cognitive flexibility test (diet $F_{(1, 48)}$= 5.010 $p<0.05$). Mice with intermittent free-choice access to CD (BE group) showed a reduced hit ratio when the active lever changed in the reversal test in comparison with the group of mice fed with *ad libitum* access to standard food (STD group). These results revealed a significant reduction in the adaptive behavioural abilities, exhibiting impaired capacities to active lever pressing shift in a reversal test. Interestingly, mice under limited access to CD and overexpressing $A_{2A}R$ showed a mild trend of improved adaptive behaviour compared to those with AAV-CTRL injection in the same diet group (**Figure 34g**). Using the three food addiction-like criteria results, we individually categorised mice as vulnerable (covering 2-3 criteria) and non-vulnerable (covering 0-1 criteria) to food addiction. Mice were considered positive for an addiction-like criterion when their score for each behaviour was equal to or beyond the 75th

percentile of the distribution of the AAV-CTRL-STD group. The same percentages of vulnerable mice (20%) were obtained for AAV-CTRL independently of the diet (BE and STD groups). However, those animals overexpressing $A_{2A}R$ exposed to intermittent free-choice access reduced from 20 to 15.38 the percentage of mice that achieved 2 or 3 criteria for vulnerability to food addiction. Of note, those animals overexpressing $A_{2A}R$ strongly decreased the likelihood to develop a vulnerability to food addiction with a total of 0% of mice that achieved more than one criterion (**Figure 34e**).



	Non-addicted		Addicted	
standard	0 crit	1 crit	2 crit	3 crit
AAV-control	40%	40%	20%	0%
AAV-A2a	81.82%	18.18%	0%	0%
binge				
AAV-control	53.33%	26.67%	20%	0%
AAV-A2a	61.54%	23.08%	15.38%	0%

Figure 34. Behavioural evaluations associated with addiction-like criteria results. a-c) Behavioral tests of the 3 addiction-like criteria show decreased motivation in AAV-A2aR mice. The dashed horizontal line indicates the 75th percentile distribution of mice treated with AAV-CTRL fed with StdF (control group). Vulnerable mice in grey filled circles for AAV-CTRL mice (STD and BE groups) and red for AAV-A2aR mice (STD and BE groups). One-way ANOVA test

(individual values with the median and the interquartile range). a) Motivation, progressive ratio test. (injection *p<0.05). b) Persistence to response, pellet free period. (injection *p<0.05. injection*diet p= 0.054 n.s). c) compulsivity, foot-shock test. d) Percentage of mice over-expressing A2aR classified as food addicted animals. e) Impulsivity test, active responses during the time out. g) Cognitive flexibility, reversal test. (diet *p<0.05). Statistical details are included in Supplementary Table S2.

A_{2a}R upregulation in the indirect ventral pathway did not modify locomotor activity either emotional and cognitive phenotype

Given the already reported effect of A_{2A}R agonist (CGS 21680) injection into the NAc decreasing locomotor activity (Barraco et al., 1993; Hauber and Münkle, 1997), we wanted to determine whether the overexpression of A_{2A}R in the NAc-VP pathway could modify the locomotor activity to avoid misleading interpretation food intake and reward-seeking behaviour evaluation. For this purpose, locomotor activity tests were performed at the beginning (before surgeries) and the end of the experiment on week 16th after surgeries. We did not find significant changes in rearing responses to measure vertical activity or in total locomotor activity between injection groups, neither before nor after surgeries (pre-surgeries: rearing groups*time $F_{(3, 45)}$= 0.868, n.s; total activity groups*time $F_{(3, 45)}$= 0.189, n.s; post-surgeries: rearing groups*time $F_{(3, 45)}$= 2.310, n.s; total activity, groups*time $F_{(3, 45)}$= 0.478, n.s) (**Figure 35a-d**).

Additionally, we investigated the possible effects of the A_{2A}R upregulation on emotional and cognitive responses. In agreement with Czyzyk, Sahr and Statnick, 2010, repeated binge-like eating episodes did not alter anxiety- or depression-like behaviour. Depressive- and anxiety-like behaviours were measured by a forced swimming test (FST) and elevated plus maze (EPM), respectively, in craving conditions for palatable food two days before the next free-choice 24 h-period. We found no

changes in the immobility time in the FST (diet $F_{(1, 52)}$= 0.215, n.s injection $F_{(1, 52)}$= 0.805 n.s; injection*diet $F_{(1,52)}$= 0.403, n.s) (**Figure 36a**), nor in the percentage of time spent in the open arms of the EPM as anxiogenic-like behaviour, comparing injection and diet groups (diet $F_{(1,46)}$= 0.541, n.s; injection $F_{(1, 46)}$= 0.180, n.s; injection*diet $F_{(1, 46)}$= 1.964, n.s) (**Figure 36b**). Similarly, we did not observe significant changes in cognitive performance when measuring both short- and long-term memory using the novel object recognition test (NORT). Independently of the diet and viral injection, we found no changes in the discrimination index measured by the NOR test (STM: diet $F_{(1, 49)}$= 0.973, n.s; injection $F_{(1, 49)}$= 0.700, n.s; injection*diet $F_{(1, 49)}$= 0.157, n.s; LTM: diet $F_{(1, 49)}$= 3.513, n.s; injection $F_{(1, 49)}$= 0.001, n.s; injection*diet $F_{(1, 49)}$= 0.436, n.s) (**Figure. 36c and d**).

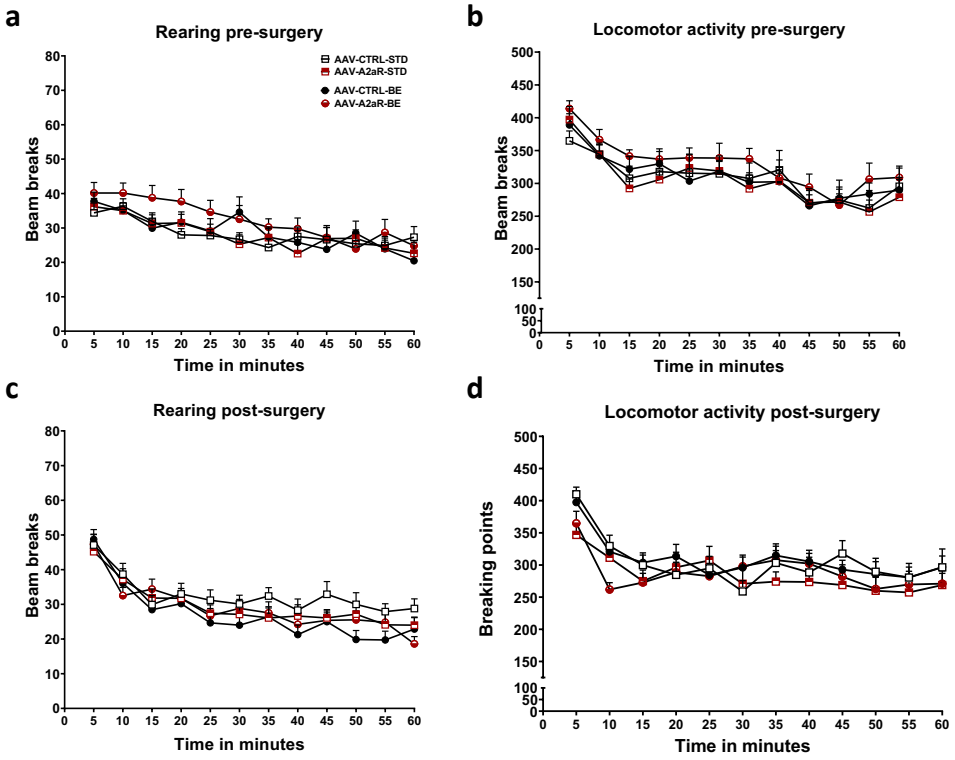


Figure 35. Locomotor activity was measured by beam breaks represented in 5-min blocks during 1 hour in both injected groups. a) Vertical locomotion activity recorder before surgeries. **b)** total lateral locomotor activity before surgeries. **c)** Vertical locomotion activity recorder after surgeries. **d)** total lateral locomotor activity after surgeries. Repeated measures ANOVA test, mean ± SEM. Statistical details are included in supplementary table S3.

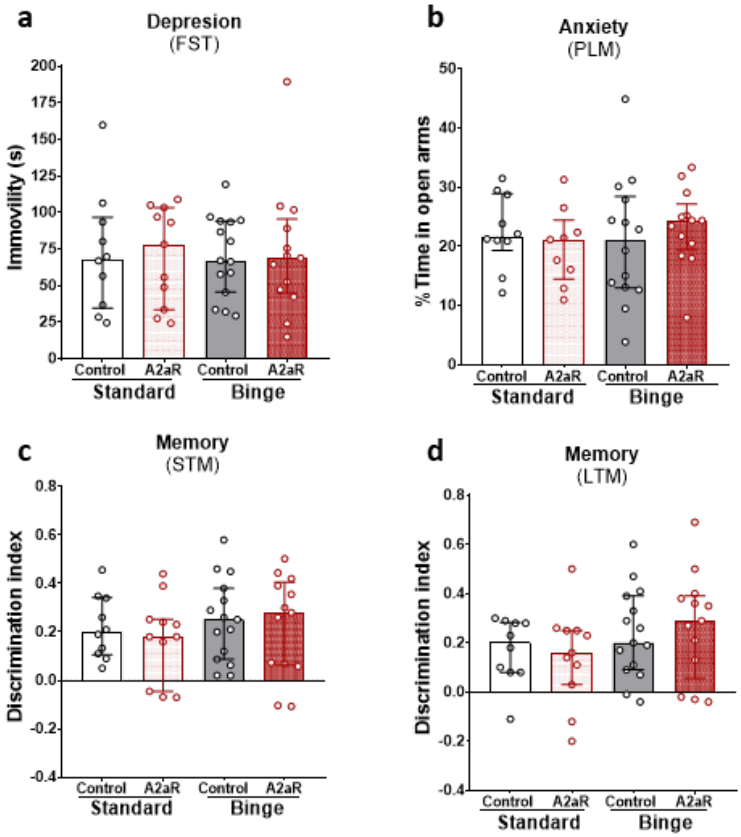


Figure 36. Emotional and cognitive test. a) Depressive-like behaviour. Immobility time of the forced swimming test**. b)** Anxiety-like behaviour. Time spent in open arms of the elevated plus-maze**. c)** Short-term memory. Discrimination index. **d)** Long-term memory. Discrimination index. One-way ANOVA test, Mean ± SEM. Statistical details are included in supplementary table S3.

Supplementary

Table S1. Model of binge-like eating behaviour. Food intake (Fig. 30-32)				
Figure number	**Statistical analysis**	**Factor name**	**Statistic value**	**P-value**
Fig. 30a-c	**Mix model test**	**STD group**		
		StdF		
		Injection_group	$F(1, 57) = 3.299$	n.s
		Time	$F(2, 57) = 835.268$	p<0.001
		Injection_group*time	$F(2, 57) = 0.272$	n.s
		BE group		
		StdF		
		Injection_group	$F(1, 78) = 0.054$	n.s
		Time	$F(2, 78) = 20.534$	p<0.001
		Injection_group*time	$F(2, 78) = 0.213$	n.s
		CD		
		Injection_group	$F(1, 77) = 0..390$	n.s
		Time	$F(2, 77)= 682.179$	p<0.0001
		Injection_group*time	$F(2, 77)= 2.441$	n.s
	Bonferroni's multiple comparison tests	**STD group**		
		AAV-CTRL vs AAV-A2aR		
		1.5 h	$F(1, 57)= 1.731$	n.s
		3 h	$F(1, 57)= 1.912$	n.s
		24 h	$F(1, 57)= 0.200$	n.s
		BE group		
		StdF		
		AAV-CTRL vs AAV-A2aR		
		1.5 h	$F(1, 78)= 0.027$	n.s
		3 h	$F(1, 78)= 0.444$	n.s
		24 h	$F(1, 78)= 0.010$	n.s
		CD		
		AAV-CTRL vs AAV-A2aR		
		1.5 h	$F(1, 77)= 0.211$	n.s
		3 h	$F(1, 77)= 0.406$	n.s
		24 h	$F(1, 77)= 4.613$	n.s
Fig. 31a-i	**Mix model test**	**STD group 1.5 h**		
		StdF		
		Injection_group	$F(1, 114) = 5.381$	p<0.05
		BC	$F(5, 114) = 1.952$	n.s
		Injection_group*BC	$F(5, 114) = 0.178$	n.s
		BE group 1.5 h		
		StdF		
		Injection_group	$F(1, 155) = 1.966$	p<0.01
		BC	$F(5, 155) = 12.304$	n.s
		Injection_group*time	$F(5, 155) = 0.207$	n.s
		CD		
		Injection_group	$F(1, 156) = 9.726$	p<0.01
		BC	$F(5, 156)= 1.852$	n.s
		Injection_group*time	$F(5, 156)= 0.252$	n.s
		STD group 3 h		
		StdF		
		Injection_group	$F(1, 113) = 0.478$	n.s
		BC	$F(5, 113)= 3.655$	n.s
		Injection_group*time	$F(5, 113)= 0.077$	n.s
		BE group 3 h		
		StdF		

		Injection_group	$F(1, 154) = 1.492$	n.s
		BC	$F(5, 154) = 0.372$	n.s
		Injection_group*time	$F(5, 154) = 0.081$	n.s
		CD		
		Injection_group	$F(1, 156) = 27.658$	p<0.001
		BC	$F(5, 156) = 2.414$	p<0.05
		Injection_group*time	$F(5, 156) = 0.260$	n.s
		STD group 24 h		
		StdF		
		Injection_group	$F(1, 114) = 2.713$	n.s
		BC	$F(5, 114) = 1.799$	n.s
		Injection_group*time	$F(5, 114) = 0.649$	n.s
		BE group 24 h		
		StdF		
		Injection_group	$F(1, 150) = 2.362$	n.s
		BC	$F(5, 150) = 0.216$	n.s
		Injection_group*time	$F(5, 150) = 0.201$	n.s
		CD		
		Injection_group	$F(1, 156) = 0.307$	n.s
		BC	$F(5, 156) = 0.352$	n.s
		Injection_group*time	$F(5, 156) = 0.870$	n.s
	Bonferroni's multiple comparison tests	**1.5 h**		
		AAV-CTRL vs AAV-A2aR		
		STD group		
		BC 5	$F(1, 114) = 1.402$	n.s
		BC 6	$F(1, 114) = 0.490$	n.s
		BC 7	$F(1, 114) = 0.300$	n.s
		BC13	$F(1, 114) = 0.804$	n.s
		BC14	$F(1, 114) = 0.454$	n.s
		BC15	$F(1, 114) = 2.823$	n.s
		BE group StdF		
		BC 5	$F(1, 155) = 1.761$	n.s
		BC 6	$F(1, 155) = 2.797$	n.s
		BC 7	$F(1, 155) = 3.563$	n.s
		BC13	$F(1, 155) = 3.442$	n.s
		BC14	$F(1, 155) = 0.456$	n.s
		BC15	$F(1, 155) = 2.403$	n.s
		BE group CD		
		BC 5	$F(1, 156) = 1.111$	n.s
		BC 6	$F(1, 156) = 0.880$	n.s
		BC 7	$F(1, 156) = 0.539$	n.s
		BC13	$F(1, 156) = 1.494$	n.s
		BC14	$F(1, 156) = 2.471$	n.s
		BC15	$F(1, 156) = 4.488$	p<0.05
		3 h		
		AAV-CTRL vs AAV-A2aR		
		STD group		
		BC 5	$F(1, 113) = 0.437$	
		BC 6	$F(1, 113) = 0.155$	n.s
		BC 7	$F(1, 113) = 0.359$	n.s
		BC13	$F(1, 113) = 0.952$	n.s
		BC14	$F(1, 113) = 1.253$	n.s
		BC15	$F(1, 113) = 0.858$	n.s
				n.s
		BE group StdF		
		BC 5	$F(1, 154) = 0.178$	
		BC 6	$F(1, 154) = 0.391$	n.s
		BC 7	$F(1, 154) = 0.001$	n.s
		BC13	$F(1, 154) = 0.184$	n.s
		BC14	$F(1, 154) = 0.366$	n.s
		BC15	$F(1, 154) = 0.787$	n.s

				n.s
		BE group CD		
		BC 5	$F(1, 156)= 4.042$	
		BC 6	$F(1, 156)= 4.999$	p<0.05
		BC 7	$F(1, 156)= 2.322$	p<0.05
		BC13	$F(1, 156)= 7.710$	n.s
		BC14	$F(1, 156)= 2.801$	p<0.01
		BC15	$F(1, 156)= 7.082$	n.s
				p<0.01
		24 h		
		AAV-CTRL vs AAV-A2aR		
		STD group		
		BC 5	$F(1, 114)= 0.00$	
		BC 6	$F(1, 114)= 2.042$	n.s
		BC 7	$F(1, 114)= 0.039$	n.s
		BC13	$F(1, 114)= 2.900$	n.s
		BC14	$F(1, 114)= 0.962$	n.s
		BC15	$F(1, 114)= 0.16$	n.s
				n.s
		BE group StdF		
		BC 5	$F(1, 150)= 0.229$	
		BC 6	$F(1, 150)= 0.808$	n.s
		BC 7	$F(1, 150)= 0.459$	n.s
		BC13	$F(1, 150)= 1.693$	n.s
		BC14	$F(1, 150)= 0.000$	n.s
		BC15	$F(1, 150)= 0.178$	n.s
				n.s
		BE group CD		
		BC 5	$F(1, 156)= 0.009$	
		BC 6	$F(1, 156)= 0.065$	n.s
		BC 7	$F(1, 156)= 0.115$	n.s
		BC13	$F(1, 156)= 1.934$	n.s
		BC14	$F(1, 156)= 1.218$	n.s
		BC15	$F(1, 156)= 1.316$	n.s
				n.s
Fig. 32a-c	**Mix model test**	**STD group**		
		StdF		
		Injection_group	$F(1, 57)= 0.018$	n.s
		time	$F(2, 57)= 5.235$	p<0.01
		Injection_group*BC	$F(2, 57)= 0.268$	n.s
		BE group		
		StdF		
		Injection_group	$F(1, 75)= 1.422$	n.s
		time	$F(2, 75)= 1.725$	n.s
		Injection_group*time	$F(2, 75)= 1.727$	n.s
		CD		
		Injection_group	$F(1, 78)= 6.687$	p<0.05
		time	$F(2, 78)= 73.698$	p<0.001
		Injection_group*time	$F(2, 78)= 1.318$	n.s
	Bonferroni's multiple comparison tests	STD group		
		1.5 h	$F(1, 57)= 0.070$	n.s
		3 h	$F(1, 57)= 4.52$	n.s
		24 h	$F(1, 57)= 0.031$	n.s
		BE group StdF		
		1.5 h	$F(1, 75)= 4.606$	p<0.05
		3 h	$F(1, 75)= 0.096$	n.s
		24 h	$F(1, 75)= 0.157$	n.s
		BE group CD		
		1.5 h	$F(1, 78)= 4.178$	p<0.05
		3 h	$F(1, 78)= 5.114$	p<0.05
		24 h	$F(1, 78)= 0.030$	n.s

Table S2. Self-administration results. Addiction-like criteria (Fig. 33 and 34)

Figure number	Statistical analysis	Factor name	Statistic value	P-value
Fig. 33	**Repeated measures ANOVA**	**Training phase FR1 (session 1-5)**		
		Session	F(1, 44)= 55.747	p<0.001
		Groups	F(3, 44)= 2.808	n.s
		groups*session	F(3, 44)= 1.519	n.s
		FR5 (session 6-12)		
		Session	F(1, 44)= 0.336	n.s
		Groups	F(3, 44)= 1.144	n.s
		groups*session	F(3, 44)= 1.540	n.s
		Experimental phase FR5 (session 13-28)		
		Session	F(1, 44)= 98.617	p<0.001
		Groups	F(3, 44)= 0.812	n.s
		groups*session	F(3, 44)= 1.144	n.s
Fig. 34	**One way ANOVA**	**Motivation test**		
		Diet	F(1, 48)= 0.088	n.s
		Injection	F(1, 48)= 5.616	p<0.05
		Diet*injection	F(1, 48)= 0.085	n.s
		Persistence test		
		Diet	F(1, 48)= 0.504	n.s
		Injection	F(1, 48)= 6.052	p<0.05
		Diet*injection	F(1, 48)= 3.920	n.s (p=0.054)
		Compulsivity test		
		Diet	F(1, 48)= 0.088	n.s
		Injection	F(1, 48)= 5.616	n.s
		Diet*injection	F(1, 48)= 0.085	n.s
		Impulsivity		
		Diet	F(1, 48)= 0.559	n.s
		Injection	F(1, 48)= 4.305	n.s (p=0.055)
		Diet*injection	F(1, 48)= 5.737	n.s
		Cognitive Flexibility		
		Diet	F(1, 48)= 5.010	p<0.05
		Injection	F(1, 48)= 0.197	n.s
		Diet*injection	F(1, 48)= 0.939	n.s

Table S3. Emotional and cognitive test (Fig. 35 and 36)

Figure number	Statistical analysis	Factor name	Statistic value	P-value
Fig. 35a-d	**Repeated measures ANOVA**	**Rearing Pre-surgery**		
		time	F(1, 45)= 55.380	p<0.001
		Groups	F(3, 45)= 0.868	n.s
		GGroupstime	F(3, 45)= 0.868	n.s

		Post-surgery		
		time	F(1, 45)= 190.064	p<0.001
		Groups	F(3, 45)= 0.892	n.s
		Groups*time	F(3, 45)= 2.310	n.s
		Total activity		
		Pre-surgery		
		time	F(1, 45)= 66.183	p<0.001
		Group	F(3, 45)= 0.830	n.s
		Groups*time	F(3, 45)= 0.189	n.s
		Post-surgery		
		time	F(1, 45)= 38.802	p<0.001
		Group	F(3, 45)= 0.722	n.s
		Groups*time	F(3, 45)= 0.478	n.s
Fig. 36a-d	**One way ANOVA**	**Depression**		
		Injection	F(1, 52)= 0..805	n.s
		Diet	F(1, 52)= 0.215	n.s
		Injection*diet	F(1, 52)= 0.403	n.s
		Anxiety		
		Injection	F(1, 46)= 0.180	n.s
		Diet	F(1, 46)= 0.541	n.s
		Injection*diet	F(1, 46)= 1.964	n.s
		Short-term Memory		
		Injection	F(1, 49)= 0.700	n.s
		Diet	F(1, 49)= 0.973	n.s
		Injection*diet	F(1, 49)= 0.157	n.s
		Long-term Memory		
		Injection	F(1, 49)= 0.001	n.s
		Diet	F(1, 49)= 3.513	n.s
		Injection*diet	F(1, 49)= 0.436	n.s

Study 3

A Gallego-Román[1], R. Maldonado[1,2*], E. Martín-García[1*], M. Martín-Sánchez[1#*].

[1]. University Pompeu Fabra, Barcelona, Spain.

[2]. IMIM (Hospital del Mar Medical Research Institute), Barcelona, Spain.

[#] Current address: Eurecat, Centre Tecnològic de Catalunya, Unitat de Nutrició i Salut, Reus, Spain.

[*] Equally contributed.

Behavioural characterisation of a mouse model of binge-like eating behaviour in PheComp cages

Mice exposed to intermittent free-choice access to cafeteria diet showed meal pattern modification

To assess whether the model of limited access to cafeteria diet is sufficient to recapitulate the features of clinical diagnostic criteria used in psychiatric manuals (American Psychiatric Association, 2013)**,** we analysed differences in eating patterns and behavioural disturbances similarly that was previously reported (Espinosa-Carrasco et al., 2018; Fructuoso et al., 2019). We used the PheCOMP cages to automatically record the food intake and activity of individualised mice. 11 WT C57BL/6J male mice were subjected to the experimental protocol depicted in Figure 37a. We calculated six different parameters using the food consumption readouts during 24 h-period to determine the feeding pattern features in basal (with ad libitum access to standard food) and aberrant feeding conditions (with limited access to high palatable food): total food intake (gr food/gr animal), total energy intake (kCal/gr animal), number of meals, eating rate (mg food/sec), the average duration of meals (sec) and satiety rate (sec/kCal).

Mice subjected to intermittent free-choice access did not significantly increase the total amount of food at the beginning of the protocol (at the second cycle of limited access to palatable food) in comparison to the baseline conditions. However, following an extended period of intermittent free-choice access, mice progressively increased the total amount of food consumed during 24 h-period in comparison to the

baseline conditions (time ($F_{(2, 20)}$= 4.208, p<0.05) (Figure 37b). This overconsumption of food was mainly caused by an increase in the total amount of food coming from high enriched and palatable food, revealing a significant increase in the total kCal/gr animal during 24 h-period of free-choice conditions in comparison with baseline (time $F_{(2, 20)}$= 14.534, p<0.001) (Figure 37c). The total number of meals was also increased progressively through the behavioural protocol development ($F_{(2, 20)}$= 12.422, p<0.001) (Fig. 14d), revealing important changes in food-seeking behaviour by the number of visits to the feeders when palatable food is available compared to basal conditions. Mice under free-choice conditions also increase the amount of food consumed per second, that is, they eat more food in a shorter period of time. However, this increase was not statically significant relative to baseline conditions (Figure 37e). On the other hand, the interval period between meals (average duration of meals) was significantly reduced compared to standard food conditions from the beginning of the protocol, showing a constant snacking when mice are under free-choice conditions ($F_{(1.180, 11.800)}$= 26.648 p<0.001) (Figure 37f). Consequently, **t**he satiety ratio, which is defined as the time elapsed until re-feeding in sec/kCal, was significantly reduced during free-choice periods (time $F_{(2, 20)}$ = 3.852, p<0.05) (Figure 37g). This result demonstrated that mice are less satiated and re-feed more frequently even if they consume more food and energy than in standard conditions.

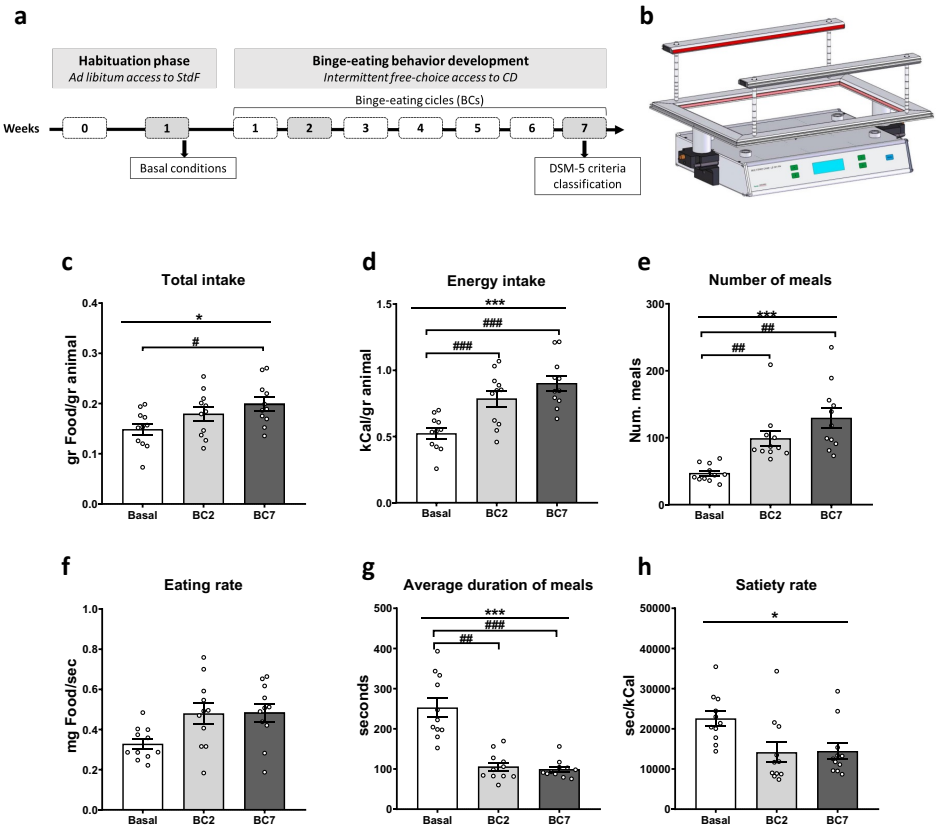


Figure 37. Prolonged exposition to intermittent free-choice access to CD cause diet-specific meal pattern modification. a) Timeline of the experimental sequence of BE behavioural model. In grey are the representative days for the calculations of meal pattern parameters. **b)** PheCOMP cages as a schematic representation. The equipment used to automatically record the food/water intake and activity of individualised mice. **c)** Total amount of food intake in 24 h at different time points expressed in gr of food/ gr of bodyweight. **d)** Total energy intake in 24 h at different time points expressed in kCal food/ gr bodyweight. **e)** Total number of meals in 24 h at different time points. **f)** binge-like eating behaviour calculated as eating rate in 24 h at different time points expressed in mg of food/sec. **g)** Average time spent on feeding during 24 h at different time points. **h)** Seconds until next meal per kCal consumed in 24 h at different time points. Data are expressed in mean ± SEM, one-way repeated measures ANOVA. Time effect *<0.05, **<0.01, ***p<0.001; Bonferroni's posthoc to determine

differences between groups #p<0.05, ##p<0.01, ###p<0.001; n=11. Basal= ad libitum access to StdF; BC= binge-like eating cycle.

Longitudinal behavioural analysis revealed cognitive inflexibility following an extended period of repetitive binge-like eating episodes

To unravel intraindividual and interindividual variability in behavioural readouts, we characterised the meal pattern disturbances leading to compulsive overeating in BE mice. Thus, we analysed the fine-grained pattern of feeding changes from basal conditions of *ad libitum* StdF to lengthy exposition to intermittent free-choice access. During basal conditions, mice showed a structured feeding pattern activity, with well-controlled daily oscillations (**Figure 38a**). All mice showed a circadian feeding pattern with higher activity in the dark phase of the cycle (active phase of the light/dark cycle) with bouts or meals well distributed with spaced times between them and almost absent during the light phase (resting phase). No patterns of preference between feeders were observed. However, when mice were exposed to intermittent free-choice access, they rapidly changed the pattern of their meals (**Figure 38b**). The bouts recorded in the CD feeder (highlighted in green) were shorter in time of duration (X-axes), and the total amount of food (Y-axes) was increased during each meal, compared to the StdF feeder (highlighted in orange). Furthermore, the frequency of meals, that is, the time elapsed until re-feeding, were shorter in the CD feeder, indicating a low satiety potential.

Using this longitudinal view of the total period of free-choice (free-choice 24 h-period), we could also demonstrate that mice challenged to this behavioural protocol modify their feeding pattern mainly during the first 3 h from CD presentation (Figure 38b). Additionally, this individual recording of food intake per second shows us that this compulsive

overeating behaviour during this time window was even exaggerated following seven cycles of intermittent free-choice exposition (**Figure 39a**).

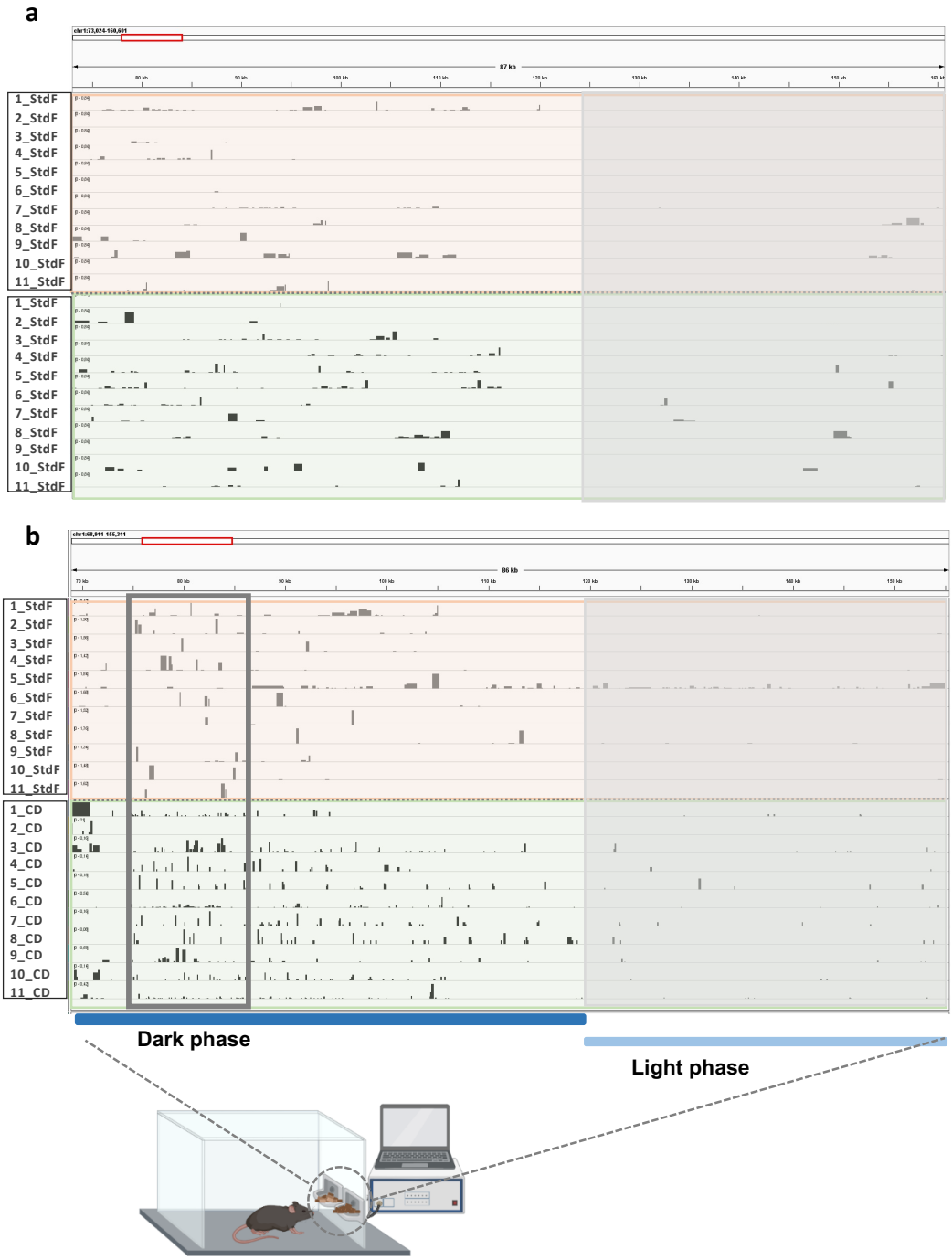


Figure 38. Representative feeding pattern during 24h. Tracks represent mouse individual feeding behaviours with feeders separated into two sections (right feeder in orange, left feeder in green. Y-axes represent the amount of food consumed, and X-axes the total time spent during a meal or bout. The light phase (resting phase) is highlighted in grey. Data represented with Pergola website and Integrative Genomics Viewer (IGV) software. **a)** Pattern of food consumption in *ad libitum* StdF conditions. **b)** Pattern of food consumption in free-choice conditions during 24h in the second cycle of binge eating (BC2). The first 3 h from the CD presentation is delimited in a dark grey square.

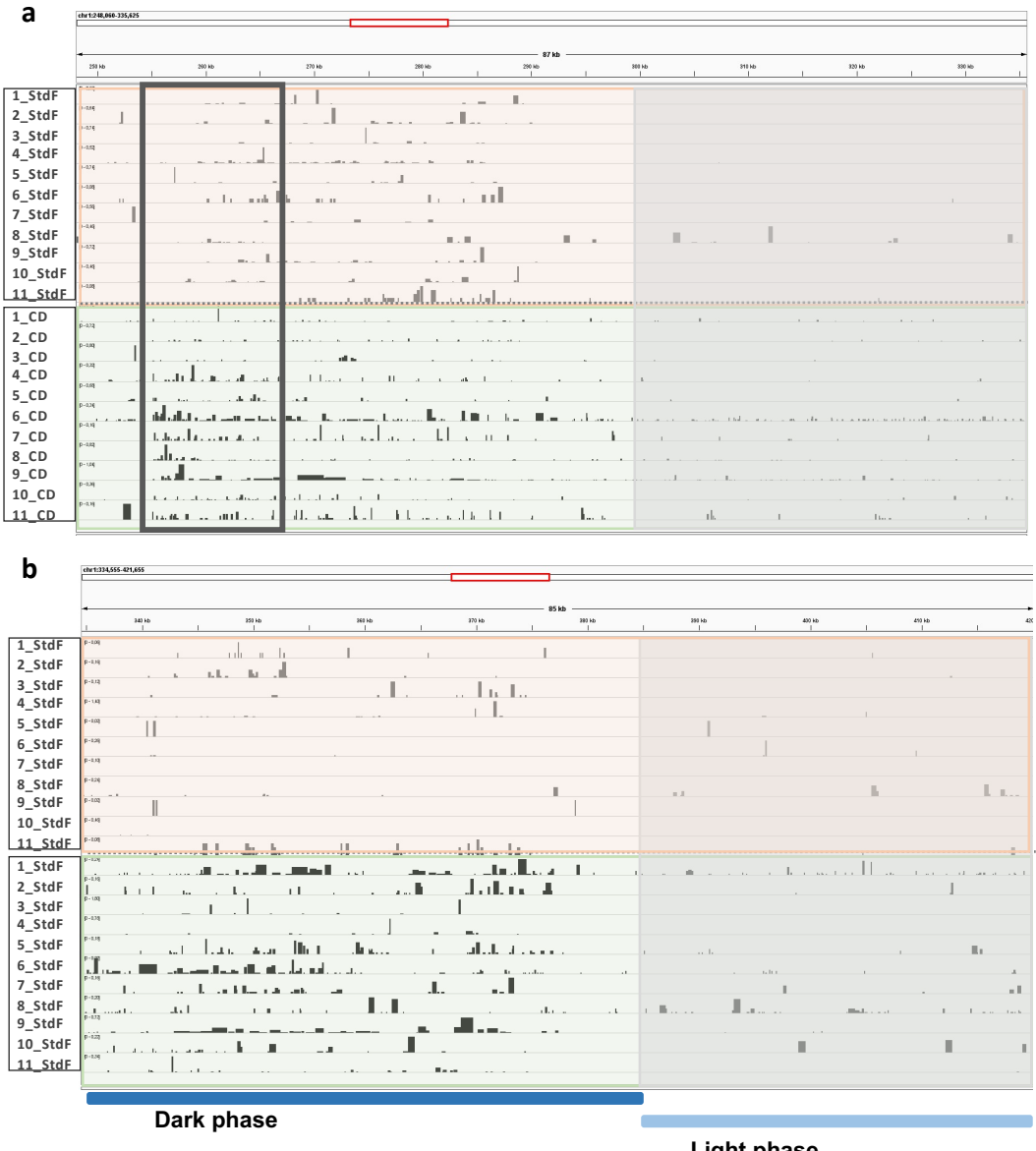


Figure 39. Representative feeding pattern during 24h. a) Pattern of food consumption in free-choice conditions in the seventh cycle of binge eating (BC7). The first 3 h from the CD presentation is delimited in a square. **b)** Pattern of food consumption in *ad libitum* StdF condition after BC7.

In humans, binge-eating disorder is associated with an increase in food and energy intake in a short period of time. However, patients with the binge-eating disorder also show increased levels of craving for palatable food (Longena and Davis, 2013, and cognitive bias associated with cognitive inflexibility (Voon, 2015), performing more set-shifting and perseverative errors compared with non-binge eaters. These alterations were also investigated in BE mice after seven weeks of intermittent free-choice exposure to develop more reproducible translational criteria in rodent models. **Figure 39** represents the free-choice 24 h-period (**Figure 39a**) together with the following 24 h-period without palatable food available (**Figure 39b**). Following each cycle of binge eating, the CD was again replaced by StdF. However, following seven cycles of conditional binge eating, individual readouts showed constant seeking in the feeder that previously contained the CD pellets one day after BC7 (**Figure 39b**). In addition, the feeding patterns with and without palatable food were substantially similar regarding the number of meals (or visits to the feeders) and the time spent on eating (average duration of meals). These two parameters were compared between feeders during the 24 h in the absence of palatable food, showing an increase seeking-like behaviour for palatable food represented as a significant increase in the numbers of visits at the feeder that previously contained the CD ($t= -4.218$, $p<0.01$) (**Figure 40a**). The time spent eating was also higher in the feeder that previously provided palatable food but was not significantly different ($t= -1.823$, n.s) (**Figure 40b**). Despite the prolonged repetition of this protocol

for seven weeks, most mice continued to forage for palatable food with maintained persistence throughout the active phase (12 h of dark phase) (**Figure 38b and 39a**).

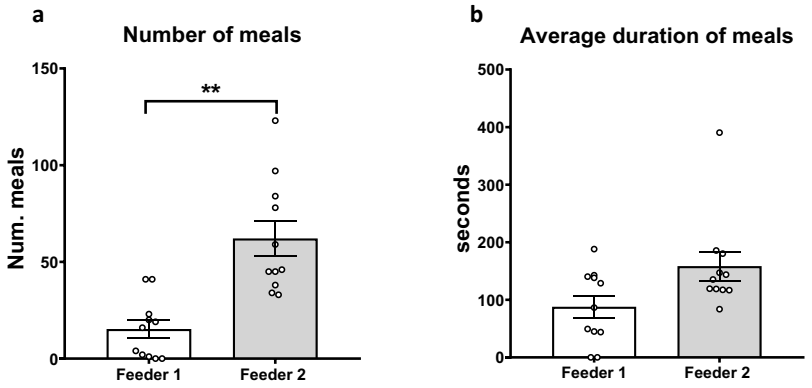


Figure 40. Comparative feeder preference. Individual values with the mean ± SEM. Paired-t-test. **p<0.01. a) Number of meals during 24h after BC7. b) Average of time spent eating in each feeder in seconds**.**

Subpopulation differentiation in vulnerable and resistant to binge-eating disorder in a heterogeneous population of mice

Binge-eating disorder is an eating disturbance listed in the latest version of the DMS-5. Thus, the diagnostic criteria used in psychiatric manuals should be considered when developing animal models to study specific eating disorders. The longitudinal and individual study using PheCOMP cages allowed us to establish five main quantifiable criteria as hallmarks for identifying mice vulnerable to binge-eating disorder in a heterogeneous population, summarised in **Figure 41**.

First, we establish the discrete period of time to determine an episode of binge-eating (the first 3 h from the BC onset). To evaluate the lack of control, we measure the eating rate in mg of food per second during this delimited period. This criterion showed us that following six weeks of

intermittent free-choice access, mice exacerbated their BE behaviour, increasing their eating rate in the first 3 h from CD presentation comparing the early period (BC2) to the late period (BC7) (**Figure 42a**) (t= -3.452, p<0.01). Then, we analysed the features of each BE episode: the eating velocity (average duration of meals. **Figure 42b**) and the abnormal amount of food consumed (food intake and the number of meals, **Figure 42d and c**), even when they do not feel hungry (energy intake, **Figure 42e**). Concerning these parameters, after six weeks of intermittent free-choice conditions exposition, mice increased the number of meals significantly in the CD feeder (t= -2.730, p<0.05) and the total amount of food consumed (t= -3.452, p<0.01).

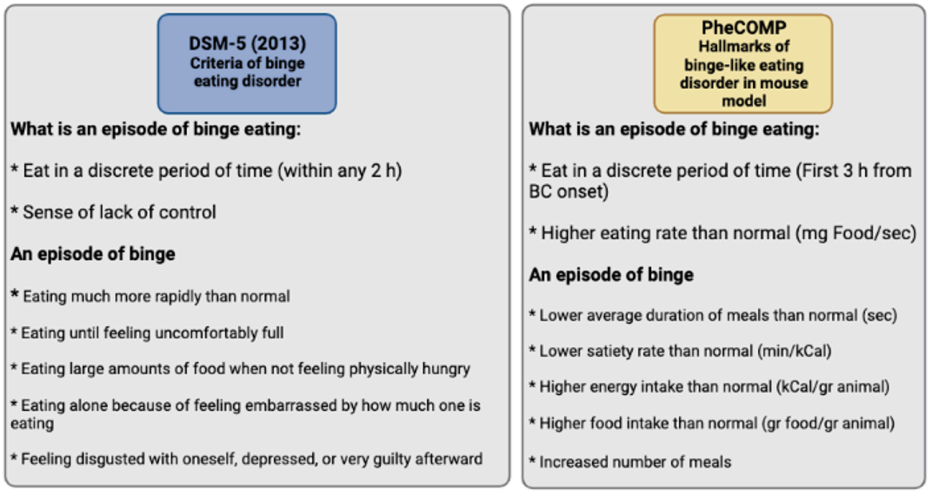


Figure 41. Diagnostic criteria of BED in the DMS-5 compared to the criteria measured in rodent models of BE.

Taking together all these parameters, the heterogeneous mice population was analysed individually. A mouse was considered positive for a binge-eating disorder criterion when its score for each parameter was equal to or major than the 75th percentile of the distribution in CD feeder in the late period. A total of 27.27% of mice achieved at least three out of

five binge-eating disorder criteria (**Figure 42f**). These data showed that although all mice exposed to intermittent free-choice access to CD developed BE behaviour, only a percentage of them met the diagnostic criteria for binge-eating disorder.

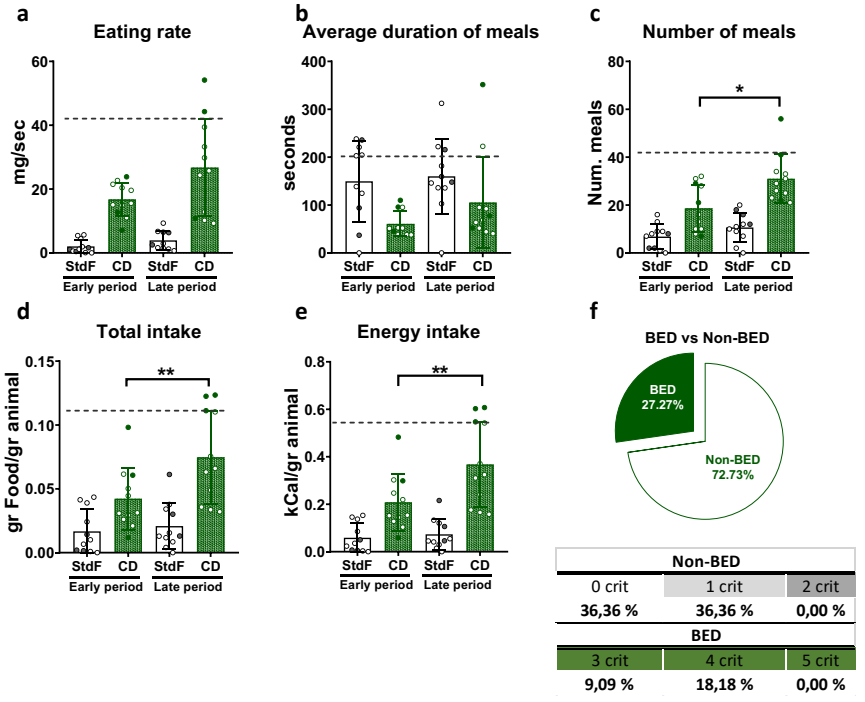


Figure 42. Diagnostic criteria of BED in a rodent model. The dashed horizontal line indicates the 75th percentile of the distribution of mice performances in the CD feeder during the BC7. Mice in green achieved at least three out of five BED criteria. Individual values with the median and the interquartile range. a) eating rate to determine binge-like behaviour (mg food/second). b) Average duration of meals to calculate the time spent eating in each feeder (seconds). c and d) total number of meals and total food intake to determine the amount of food consumed from each feeder in 3 h and the number of bouts. e) Total of energy intake in 3 h from each feeder to determine food consumption despite being satiated. f) Subpopulation differentiation of BE mice into mice that achieved at least three BED criteria (mice with BED) and those that achieved less than three BED criteria (resilience population). BC2= early period; BC7=late period; BED= Binge-eating disorder.

Supplementary

Table S1. Emotional and cognitive test (Fig. 37 and 40)				
Figure number	**Statistical analysis**	**Factor name**	**Statistic value**	**P-value**
Fig. 37 b-g	One-way repeated measures ANOVA	**Energy intake**		
		Time	F(2, 20)= 14.534	p<0.001
		Total intake		
		Time	F(2, 20)= 4.208	p<0.05
		Num. meals		
		Time	F(2, 20)= 12.422	p<0.001
		Eating rate		
		Time	F(2, 20)= 3.320	n.s
		Average duration meals		
		Time	F(1.180, 11.800)= 26.648	p<0.001
		Satiety rate		
		time	F(2, 20)= 3.852	p<0.05
	Bonferroni's posthoc	**Energy intake**		
		Basal vs BC2	-0.263 (95% CI, -0.444 to -0.081)	p<0.01
		Basal vs BC7	-0.379 (95% CI, -0.566 to -0.191)	p<0.01
		BC2 vs BC7	-0.116 (95%CI, -0.361 to 0.129)	n.s
		Total intake		
		Basal vs BC2	-0.031 (95% CI, -0.077 to 0.15)	n.s
		Basal vs BC7	-0.05 (95% CI, -0.099 to -0.002)	p<0.05
		BC2 vs BC7	-0.02 (95% CI, -0.076 to 0.036)	n.s
		Num. meals		
		Basal vs BC2	-51.818 (95%CI,-87.371 to -16.265)	p<0.01
		Basal vs BC7	-82.455 (95% CI, -129.649 to -35.261)	p<0.01
		BC2 vs BC7	-30.636 (95% CI, -89.116 to 27.843)	n.s
		Eating rate		
		Basal vs BC2	-0.151 (95%CI,-0.353 to -0.051)	n.s
		Basal vs BC7	-0.156 (95% CI, -0.314 to 0.002)	n.s p=0.054
		BC2 vs BC7	-0.005 (95% CI, -0.231 to 0.221)	n.s
		Average duration meals		
		Basal vs BC2	-146.846 (95%CI,58.079 to 235.612)	p<0.01
		Basal vs BC7	153.406 (95% CI, 83.432 to 223.381)	p<0.001
		BC2 vs BC7	6.561 (95% CI, -27.753 to 40.875)	n.s
		Satiety rate		
		Basal vs BC2	8405.966 (95%CI, -2064.331 to 18876.262)	n.s
		Basal vs BC7	8121.184 (95% CI -634.915 to 16877.284)	n.s
		BC2 vs BC7	-284.781 (95% CI, -10580.923 to 10011.360)	n.s
Fig. 40	Paired t-test	**Num. meals**	t= -4.218	p<0.01
		Average duration meals	t= -1.823	n.s

Table S1. Emotional and cognitive test (Fig. 42)				
Figure number	**Statistical analysis**	**Factor name**	**Statistic value**	**P-value**
Fig. 42 a-e	Paired t test	**Eating rate CD feeder**		
		BC2 vs BC7	t= -3.452	p<0.01
		Average duration meals CD feeder		
		BC2 vs BC7	t= -1.908	n.s
		Num. meals CD feeder		
		BC2 vs BC7	t= -2.730	p<0.05
		Total intake CD feeder		
		BC2 vs BC7	t= -3.452	p<0.01
		Energy intake CD feeder		
		BC2 vs BC7	t= -1.869	n.s

DISCUSSION

The overall purpose of this Doctoral Thesis was to characterize the transcriptional changes in NAc D2(+) neurons and neuronal pathways in the brain's reward system that could unravel the neuropathological mechanisms involved in the compulsive-like behaviour over food intake described in the binge-eating disorder. Nowadays, the limited availability of preclinical models to evaluate the individual diversity in humans stymies the progress in developing treatments strategies for abnormal eating behaviours. Thus, we also proposed an improved version of previous rodent models in compulsive-like eating that recapitulates most of the symptoms described in psychiatric manuals, allowing to distinguish different subpopulation of vulnerable and resistant to binge-eating disorder.

Binge-like eating behaviour is associated with alterations in the transcriptomic profile of NAc D2(+) neurons

The easy access to hypercaloric and palatable foods in Western societies is a major factor contributing to the development of abnormal eating habits that may lead to a variety of forms of uncontrolled eating. Binge-eating disorder is one of these eating disturbances, often comorbid with overweight and obesity (Smink, van Hoeken and Hoek, 2012). Despite the high comorbidity with obesity, the binge-eating disorder also may occur in non-obese persons (Wonderlich et al., 2009; Marcus and Wildes, 2012) and is associated with a higher risk of developing heart disease, stroke, type 2 diabetes, and cancer compared with people on the same overweight or obesity range without the binge-eating disorder (Kessler et al., 2013; Raevuori et al., 2015; Mitchell, 2016).

One of the particularities of this abnormal eating behaviour is the lack of control during episodes and negative emotional states with one-self and marked distress regarding binge eating afterwards as negative consequences. These behavioural features describe a compulsive-like behaviour over food intake, which would imply problems in self-control and reward-related processes. In this regard, it has been proposed that compulsive-like eating behaviour may be caused by a hypersensitivity to reward (Davis C et al., 2007), emphasizing the importance of the reward circuit as a potential target for research into susceptibility binge-eating disorder. In this last sense, one of the substantial differences between obesity and binge-eating disorder has been associated with hypo- and hyper-sensitivity to reward that may confer risk in the dynamic process of overeating, respectively (Davis et al., 2009).

Similar that occurs in drug addiction, the dopamine type-2 receptor (D_2R) in the striatal areas plays a major role in the vulnerability for compulsive consumption (Volkow, Wang and Baler, 2011; Wang et al., 2011b; Volkow, Wise and Baler, 2017). For instance, positron emission tomography (PET) imaging with [^{11}C]raclopride from obese individuals showed significantly lower levels of striatal D_2R (Volkow, Wang, Fowler, et al., 2008) and a lack of the normal increase in striatal dopamine (DA) during consumption of calories compared to the non-obese group (Gene Jack Wang et al., 2014). In this respect, the reduced sensitivity (hyposensitivity) of the DA reward system and the attenuated DA response to the reinforcer generate a mismatch between the experiences of the expected and the actual reinforcer that may lead to an overeating disturbance (Volkow, Wise and Baler, 2017). Contrary to the reported results from obesity, binge-eating disorder reveals a possible

hypersensitivity to rewards (Davis et al., 2009) that could also lead to compulsive food consumption. Genotyping studies using different single nuclei polymorphisms (SNP) for D_2R revealed that binge-eating disorder is distinguished by a greater density of D_2R and higher D2 binding potential than obese with and without binge-eating disorder (Davis et al., 2008, 2009, 2012). In addition, PET-[^{11}C]raclopride studies in groups of obese patients showed enhanced striatal DA levels during food stimulation in those also suffering from the binge-eating disorder, but not in non-binge eater individuals (Wang et al., 2011b).

Based on this previous information, the present Thesis aimed to decipher the molecular mechanisms underlying the binge eating phenotype, particularly in D2(+) neurons in the NAc, to identify biomarkers differently expressed during uncontrolled food intake. For this purpose, we compared the NAc transcriptomic profile of D2-Cre:Ribotag transgenic mice that allowed the selective expression of HA-tag in Drd2-containing cells (Sanz et al., 2009; Puighermanal et al., 2020) among three different dietary conditions. The non-obese binge-like eating group of mice with intermittent free-choice access to cafeteria diet (BE group) was compared to a diet-control group (STD group) to investigate the differences in the translatome profile during binge eating episodes and healthy diet conditions. On the other hand, we also investigated the differential effect in gene expression between prolonged repetition of binge eating and obesity conditions by comparing mice with intermittent access to highly palatable and caloric food (BE group) and those with free-choice access (FC group).

In agreement with previous studies (Czyzyk, Sahr and Statnick, 2010; Gutiérrez-Martos et al., 2018), mice subjected to intermittent access

to high caloric and palatable food conditions showed the principal phenotypic traits of binge-eating disorder, consuming abnormal amounts of energy (around 15% of total daily caloric intake) in a short period of time (1.5 h) in comparison with those obese-like fed and standard mice (**Figure 24b**). This significant increase in energy intake was due to an overconsumption of high caloric and palatable food (**Figure 24c**) that was not observed at the end of the free-choice access period (following 24 h from the binge-like eating cycles) (**Figure 24d**). Based on these behavioural results, we considered the 1.5 h-period of the binge-like eating cycle to be the most interesting moment to investigate the gene expression changes explicitly produced during binge-like eating episodes at transcriptomic level.

An overall analysis of transcriptomic changes among diet groups revealed a differential cluster distribution where binge-like eating mice differed from both obese-like fed and the standard mice groups (**Figure 25a**). This result suggested that the conditional binge-like eating episodes by intermittent free-choice access to highly caloric and palatable food were the main source of variance (61%) differing from those with prolonged exposure to *ad libitum* free-choice access. In addition, transcriptomic profiling of D2(+) neurons in the NAc revealed an upregulation of the *Drd2* gene in the binge-like eating mice compared to those fed with *ad libitum* access to standard food (**Figure 25b**) or with free-choice access to cafeteria diet (**Figure S2**). Further quantitative real-time PCR analysis to validate the RNA sequencing results confirmed that the relative mRNA expression of *Drd2* was increased in mice subjected to repetitive binge-like eating cycles while it was reduced in obese-like fed mice (**Figure 25g**). These results indicated that repetitive binge eating

might produce changes in DA signalling, which would be in agreement with previous studies in humans supporting the view that the binge-eating disorder is a condition that may have its causal origins in a hypersensitivity to reward or a "reward surfeit syndrome" (Davis C et al., 2007), in contrast to the hyposensitivity to reward reported in obese without the binge-eating disorder.

Concretely, the differential expression analysis revealed that from over 20.000 genes expressed in D2(+) neurons, 498 (2.3%) were significantly (FDR<0.05) upregulated in the binge eating group compared to the standard group, while 396 genes (1.8%) were downregulated. On the other hand, 744 genes (3.4%) were significantly upregulated, and 750 downregulated (3.4%) when binge eating and obese-like fed mice were compared. Interestingly, genes such as the *Gabrg1* (gamma-aminobutyric acid receptor subunit gamma-1) and *Ndn* (necdin protein) were downregulated, suggesting changes in GABA-mediated signalling and genetic disorders that cause hyperphagia. On the other hand, gene ontology (GO) term enrichment and gene-concept network analysis in the NAc D2(+) neurons demonstrated that the differentially expressed genes (DEGs) in binge-like eating mice were functionally associated with neuronal biological processes related to memory, cognition, and learning, among others (**Figure 25c and Figure S2b**). For example, the translatome analysis revealed a downregulation of *Atad1* encoding for ATPase Family AAA Domain Containing 1, whose absence has been associated with a decreased AMPA receptors internalization, leading to physiologic outcomes result in deficits in learning and memory (Mancebo et al., 2012). On the other hand, *Vdac3* encoding for Voltage-Dependent Anion Channel 3 was significantly downregulated in binge eat mice compared to both

standard and obese-like fed mice. VDACs have long been proposed to be a part of the Mitochondrial Permeability Transition Pore complex (MPTP) (Szabó, Pinto and Zoratti, 1993; Crompton, Virji and Ward, 1998), which are implicated in learning and synaptic plasticity based upon its role in mitochondrial calcium buffering, ATP production, and metabolite flux at the synapse (Ichas, Jouaville and Mazat, 1997; Levy et al., 2003). These findings at the transcriptomic level supported the previously reported cognitive biases in humans with binge-eating disorder. Comparative assessments between obese with and without binge-eating disorder revealed that those obese that binge eat make risky decisions significantly more often than those who do not (Svaldi, Brand and Tuschen-Caffier, 2010). Additionally, obese that binge eat also showed impaired capacities to advantageously utilize feedback processing, as well as significantly more set-shifting and perseverative errors in cognitive flexibility and inhibition control tests (Duchesne et al., 2010; Svaldi, Brand and Tuschen-Caffier, 2010; Balodis et al., 2013). These results from human assays revealed significant decision-making and executive function impairments, indicating reduced self-control in obese patients who also suffer from binge-eating disorder. However, our study revealed that in our experimental conditions, these cognitive impairments are particular to the repetitive binge-eating behaviour without obesity comorbidity.

Another outstanding result from our transcriptomic analysis was the higher enriched scores in the addiction-related pathways when we deeply compare functional alterations of the DEGs in the NAc D2(+) neurons in binge-like eating mice relative to the standard and obese-like fed mice (**Figure 25d-f and Figure S4**). These findings suggested that the repetitive binge-like eating episodes exposition was enough to stimulate

the brain reward system similar to previously reported in substance use disorders (Davis and Carter, 2009; Avena and Gold, 2011; Davis et al., 2011). This evidence highlights the complex and polyfactorial nature of binge-like eating and prompted us to focus of molecular targets particularly involved in the regulation of the brain reward system. The comparison of the transcriptomic profiles in the NAc D2(+) neurons between binge-like eating and standard mice revealed a significant upregulation of the *Cck, Mgll, Drd2* and *Adora2A* genes in binge-like eating mice (**Figure 25b**). Similarly occurred when we compared the transcriptomic profiles between binge-like eating and obese-like fed mice (**Figure S2**). All these genes are involved in the neurobiological pathways recruited in addiction-related behaviours (le Merrer et al., 2012) and food intake regulation (Nowend et al., 2001; Nagel et al., 2003; Bellocchio et al., 2010; D'Agostino et al., 2016). Cholecystokinin (CCK) neurotransmitter, encoded by *Cck*, is abundant in the NAc and colocalized with DA in afferent terminals in this region from the VTA (Pernow B, 1980) and substantia nigra (Lança et al., 1998), modulating accumbal DA turnover (Dumbrille-Ross and Seeman, 1984; Vaccarino, 1994) to regulate DA-mediated behavioural responses (Crawley, Hommer and Skirboll, 1985; de Witte et al., 1987; Kariya, Tanaka and Nomura, 1994). Thus, CCK receptors (CCK_A and CCK_B) are widely distributed in the brain and are abundant in the NAc (Crawley, 1992; Vaccarino, 1994; Mercer et al., 2000). However, the CCK_B receptor subtype is the most abundant in the CNS, to a lesser extent in the gastrointestinal tract (Innis and Snyder, 1980), while CCK_A is primarily located in the gastrointestinal tract and lesser extended in the CNS (Sankaran et al., 1980). Among the roles attributed to the activation of CCK receptors are the involvement in anxiety (van Megen et al., 1996; Koszycki

et al., 2012) with reported anxiogenic effects of CCK mediated by CCK_B (Harro, Vasar and Bradwejn, 1993), and anxiolytic effects mediated by CCK_A antagonist (Ballaz et al., 1997). CCK receptors are also involved in reward-related behaviours (Vaccarino, 1994; Rotzinger and Vaccarino, 2003) and satiety, being all these attributes essential targets for treating eating disorders, particularly binge-eating disorder.

On the other hand, monoglyceride lipase, MGLL encoded by *Mgll*, is the primary enzyme responsible for the metabolism of 2-arachidonoylglycerol (2-AG) in the brain, mainly found in presynaptic terminals (Dinh et al., 2002; Kano et al., 2009; Shu-Jung Hu and Mackie, 2015; Lu and MacKie, 2016). The hyperactivity of the endocannabinoid system (ECS) has been associated with obesity due to its important role in regulating the hedonic aspects of feeding (di Marzo and Matias, 2005). Furthermore, D_2R and $A_{2A}R$ (Adenosine A2 receptor), encoded by *Drd2* and *Adora2A*, respectively, are colocalized in the GABAergic medium-spiny neurons (MSNs) of the NAc, mainly projecting to the ventral pallidum in the indirect pathway ("No Go") (Kupchik et al., 2015; Kupchik and Kalivas, 2017b; Klawonn and Malenka, 2018). Here, $A_{2A}R$ plays a critical role in behavioural control (Ferré, 1997) by forming a heteromeric complex with D_2R with opposite functioning (Svenningsson et al., 1999). Several studies indicated the role of $A_{2A}Rs$ in locomotor response (Turgeon et al., 1996; Rimondini et al., 1997), drug discrimination tasks (Justinova et al., 2003, 2009), reward-seeking behaviour (Knapp et al., 2001; Wydra, Gołembiowska, et al., 2015), and withdrawal syndrome in rats self-administering cocaine (Wydra, Suder, et al., 2015).

Thus, according to our transcriptomic analysis results, we selected three different candidate targets based on their importance in modulating

reward-related behaviours and food intake, as well as the availability of specific pharmacological tools to modulate these targets (selective agonist and antagonist) that would allow us to carry out specific pharmacological approaches: CCK receptors, endogenous regulation of ECS and the adenosine $A_{2A}R$. Then, we validate their potential therapeutic function in reducing binge eating behaviour by modulating the activity of NAc neuron "mimicking" the results observed at the transcriptomic level.

A specific pharmacological approach to different candidate targets for modulating binge eating

One of the most differentially expressed genes found in our study was the *Cck* in binge-like eating mice compared to standard and obese-like fed mice, indicating a possible association of this neuromodulator with the regulation of the compulsive-like eating exhibit during limited free-choice access to cafeteria diet. Although the CCK receptors are abundant in the NAc with a rostrocaudal distribution (Crawley, 1992; Vaccarino, 1994; Mercer et al., 2000), the CCK neurotransmitter synthesis in the NAc by projecting neurons has not been reported so far. Thus, we first investigated if the increase in *Cck* transcript levels reported in our translatome analysis of D2(+) neurons might be synthesized by D2(+) cholinergic interneurons located in the NAc (Alcantara et al., 2003; Lewis et al., 2020) to regulate binge eating by reward-related behaviours (Rotzinger, Bush and Vaccarino, 2002; Rotzinger and Vaccarino, 2003) acting on NAc CCK receptors. According to this hypothesis, we mimic the increased CCK levels by a bilateral local infusion of cholecystokinin octapeptide sulphated (CCK-8S), a CCK agonist for CCK_A and CCK_B receptor, performed immediately before the cafeteria diet presentation.

CCK receptors activity modulation has been associated with anxiety induction. Concretely, bilateral injections of CCK-8S into the ventral hippocampus decreased the percentage of time and entries in open arms, as representative anxiogenic-like behaviour, in the elevated plus-maze test of anxiety (Moghaddam, Hosseini and Roohbakhsh, 2012). According to these previous results in other brain areas, the possible anxiety-induction by NAc CCK-8S bilateral infusion was evaluated in a preliminary study, together with locomotor activity and Irwin test to determine possible behavioural effects that may influence the food intake results interpretation. We did not observe significant changes in any parameter measured in the preliminary study (**Figure 26b, e and h**). Once no side effects were detected, the effect of NAc CCK receptor activation in regulating compulsive-like eating was investigated using two doses, 1ng/side and 2ng/side. In accordance with previous studies (Blevins, Stanley and Reidelberger, 2000), the intra-NAc CCK-8S infusion did not alter the feeding pattern in mice with *ad libitum* access to standard food. However, similar results were obtained from mice exposed to intermittent free-choice access at all measurement points, independently of the dose and type of food (**Figure 27c, f and i**). In this regard, according to the results from both approaches, we proposed that NAc D2(+) neurons may synthesize the CCK neurotransmitter to regulate binge eating in a reward-related manner. Nevertheless, its effect acting directly in the NAc CCK receptors was insufficient to cause food intake changes. Therefore, further researches are needed to explore the hypothesis of possible CCK synthesis by D2(+) MSNs, which would imply a possible role of CCK in modulating binge eating behaviours in NAc projecting areas.

The endogenous regulation of the endocannabinoid system was investigated as a candidate target according to the transcriptomic analysis results and its important reported role in regulating the hedonic aspects of feeding (di Marzo and Matias, 2005). High-calorie food enriched with high sugar and fat contents sparks the DA release in the NAc similar to drugs of abuse consumption (Hoebel et al., 1989; Geiger et al., 2009), leading to overlapping neuroadaptations in the reward circuitry that alter the motivation to obtain a reinforcer (Lutter and Nestler, 2009). This effect is caused by the high palatability of this type of food that enhances its consumption by hedonic mechanisms that prevail over caloric necessities, constituting an endogenous orexigenic signal that modulates the palatable food-evoked DA release (Solinas et al., 2006; Melis et al., 2007). This system has been widely studied in the forebrain as a crucial target for regulating food intake. Particularly, an inverse agonist drug for the cannabinoid type-1 receptor (CB_1R), rimonabant, has been used as a pharmacological approach to treat obesity (Bray and Ryan, 2007) to reduce the hyperactivity of the ECS by an opposite effect on the target cells. However, the direct interaction in CB_1R resulted in the incidence of psychiatric severe adverse events (Taylor, 2009) that led to its withdrawn from clinical use. Thus, alternative strategies based on reducing CBR activity are currently investigated.

Our DEGs analysis revealed significantly increased levels of *mgll* gene encoding the MGLL enzyme in binge-like eating mice compared to both obese-like fed and standard mice, suggesting an adaptative mechanism to reduce the hyperactivity of the ECS by reducing the endocannabinoid (eCBs) signalling (2-AG levels) during the binge-like eating episodes. Previous studies report that the administration of 2-AG in

the NAcShell cause dose-related increases in food intake (Kirkham et al., 2002), while transgenic mice with reduced 2-AG levels in the forebrain showed a decreased fat preference (Wei et al., 2016). On the other hand, decreased levels of this eCB ligand have been reported in the NAc of obese-like fed rats, consistent with the local decrease in diacylglycerol lipase (DAGL) transcripts (a key enzyme in the biosynthesis of 2-AG) (Bourdy et al., 2021). These results were discussed suggesting that the decrease in 2-AG levels following 6-weeks of free-choice access to a highly caloric diet could reflect an endocannabinoid-mediated compensatory mechanism that decreases palatable food-induced hyperphagia and restore "homeostatic" feeding (Bourdy et al., 2021). However, our transcriptomic analysis from the NAc D2(+) neurons reported significantly increased levels of MGLL in binge-like eating mice compared to obese-like fed and standard mice, suggesting a possible down-regulation of 2-AG levels in binge-like eating mice during the compulsive eating episodes relative to both obese-like fed and standard mice. The experimental conditions may explain these seemingly contradicting results and when we took the samples, following 1.5 h from the 6^{th} binge-like eating cycle onset. First, in our experimental condition, we selectively analyzed the mRNA translated in a specific cell type, the NAc D2(+) neurons, which may lead to different results than an analysis of transcript levels without cell type selection. On the other hand, during the 1.5h-period, mice from the binge-like eating group consumed an abnormal amount of highly caloric and palatable food (**Figure 24c**), which can trigger molecular signals to decrease 2-AG levels to restore the homeostatic feeding (Kirkham et al., 2002) by increasing MGLL synthesis. Considering these results, the endogenous regulation of 2-AG levels in the NAc became an interesting

target to investigate the reduction of excessive consumption of high-caloric foods through the modulation of the hedonic sense evoked by the palatability of this kind of food. Thus, we investigated the potential role of endogenous depletion of 2-AG levels in the NAc for modulating binge eating by local infusion of O-7460, a selective inhibitor of 2-AG biosynthesis via DAGLα.

First, possible subjectives effects, locomotor activity and anxiety signs following a high dose of O-7460 (10ng/side) by local injection intra-NAc were evaluated to rule out misinterpretation in assessing energy consumption. We did not observe adverse effects during the Irwin test, nor significant alterations in locomotor activity or anxiety behaviour (**Figure 26d, g and j**). Once no side-effects were detected, we investigated the potential role of a single dose of 10ng/side injection of O-7460 intra-NAc in reducing binge eating. The DAGLα inhibition resulted in a significant increase in consumption of standard food in mice belonging to the binge-like eating group compared to the baseline conditions, but not in those fed with *ad libitum* access to standard food during the first 1.5 h from the binge-like eating cycle onset and drug infusion (**Figure 27e**). Following 3 h, mice exposed to intermittent free-choice access conditions reduced significantly the total amount of kCal/gr animal consumed from highly palatable food respected to the vehicle injection, without changes in the feeding pattern in the standard group (**Figure 27h**).

Interestingly, after 24 h from the O-7460 local injection and cafeteria diet presentation, mice with intermittent free-choice conditions significantly reduced the total amount of energy intake coming from the cafeteria diet, together with an increase in the energy intake from standard food in comparison to the baseline conditions with the vehicle

injection to compensate the total daily caloric intake in 24 h-period (**Figure 27k**). No changes in the feeding pattern were detected in the diet-control group relative to the vehicle injection at any time point. These results suggested a possible reduction of the hedonic impact of palatable food as a consequence of a decreased CBRs activity (Ruiz de Azua et al., 2021) through the modulation of endogenous 2-AG levels, rather than a simple satiety effect since the principal effect was only observed in binge-like eating mice. Altogether, the transcriptomic analysis and pharmacological method underlined the importance of an alternative approach based on endogenous regulation by lowering levels of eCBs as a promising research strategy to decrease CBRs activity to treat disorders associated with overeating.

Binge-eating disorder is associated with an increased DA response to the reinforcer (Wang et al., 2011b). Therefore, the concomitant increase of *Adora2a* transcript levels together with *Drd2* in mice exposed to repetitive binge-like eating episodes compared with both obese-like fed and standard mice may be considered an adaptive compensatory mechanism to modulate DA-mediated motivation towards highly reinforcing food (Salamone et al., 2007; Font et al., 2008; Mingote et al., 2008) to restore the homeostatic feeding. To validate this hypothesis, the effect of NAc A_{2A}R activation in reducing palatable food-seeking was investigated by local infusion of CGS 21680, a specific A_{2A}R agonist, immediately before the cafeteria diet presentation.

Several studies indicated the role of A_{2A}Rs in the locomotor effects of drugs of abuse. For example, hyperlocomotion induced by acute cocaine administration can be decreased by CGS 21680 injection even in well-habituated rats to cocaine (Filip et al., 2006). Similarly, studies using

transgenic rats overexpressing $A_{2A}R$ reported that a single injection of cocaine did not increase locomotion in transgenic rats as observed in the wild-type group (Czyzyk et al., 2011). Although locomotor activity response is sometimes used to indicate a propensity for psychostimulant self-administration, some authors argue that locomotor activity is not a predictor of self-administration but the individual differences in learning and environmental novelty (Mitchell, Cunningham and Mark, 2005). Based on these results, we evaluated the locomotor activity effect duration following NAc $A_{2A}R$ activation by 1 and 2ng/side CGS 21680 infusions in a preliminary study. Local stimulation of NAc $A_{2A}R$ resulted in a depressive effect in locomotor activity in agreement with previous pharmacological studies (Barraco et al., 1993) at 1 and 2ng/side doses (**Figure 26c and Figure S9**) during the first 75 and 90 mins from the local injection. Thus, particular attention was paid to the diet-control mice in the first two time points (1.5 h and 3 h following local infusion and cafeteria diet presentation) while evaluating the palatable food-seeking effect due to the possible locomotor bias. The anxiety and the Irwin test were also evaluated to determine possible side-effects. We did not observe subjective effects in blind conditions during Irwin test following doses administration nor significant effect in anxiety behaviour (**Figure 26i**)

In the pharmacological validation, bilateral local infusion of a dose of CGS 21680 1ng/side did not alter the feeding pattern compared to baseline conditions, despite the reported depressive locomotor effect and regardless of measurement times. On the other hand, bilateral local infusion of a dose of 2ng/side resulted in a significant decrease in energy intake from the palatable food in the binge-like eating group relative to the basal condition, after 1.5 h, 3 h and 24 h from the binge-like eating

cycle onset (**Figure 27d, g and j**). Additionally, this group of mice exposed to intermittent free-choice access did not reveal alteration in the amount of kCal consumed from the standard food, independently of the time point. These data suggested that the local activation of $A_{2A}R$ with 2ng/side of CGS 21680 prevented the compulsive-like eating for palatable food by reducing the frequency and duration of the binge eating, similar to the previously reported effect following D_2R blockade in the NAcShell in rats with intermittent access to sucrose solution (Suárez-ortiz, Cortés-salazar and Malagón-carrillo, 2018). Nevertheless, significant changes were detected in the total energy intake after 3 h from 2ng/side infusion of CGS 21680 in the diet-control group compared to the baseline conditions. This significant effect in standard food intake was not detected at 1.5 h following the local infusion and cafeteria diet presentation and disappeared in the third measure (at 24 h time point). Based on the results from both transcriptomic analysis and pharmacological validation, we suggested that $A_{2A}R$ may be involved in regulating binge eating by opposite interaction with D_2R, reducing the DA-mediated reward-seeking behaviour towards high palatable food. However, our data were not conclusive due to the reported depressive effect in locomotor activity, and the food intake reduction also observed in the diet-control group. Thus, further investigation discussed in the next section was performed to investigate the role of $A_{2A}R$ in the NAc indirect pathway to reduce palatable food intake.

Altogether, these findings demonstrated similarities across differing pathological disorders, such as drug addiction and binge-eating disorder, which provides insights into dimensional mechanistic similarities that might cut across seemingly differing behaviours. The significant

increase in the transcript levels of *Cck* suggested the synthesis of this neuromodulator by D2(+) neurons in the NAc, which to our knowledge, has not been reported so far. These results indicated a possible role of CCK in modulating binge eating in a reward-related manner, probably by affecting in NAc projecting areas. Additionally, the functional validation by the pharmacological approach remarked the importance of the ECS in regulating food intake in a hedonic sense and how reducing the endogenous levels of 2-AG could be an alternative approach for more accurate treatments of eating disorders that implicate overeating. On the other hand, the NAc $A_{2A}R$ activation by 2ng/side of CGS 21680 in our experimental conditions reduced energy intake from palatable food. However, the reported effect over locomotor activity, resembling those of DA antagonists (Wardas, Konieczny and Pietraszek, 2003), did not allow conclusive results.

Adenosine A_{2A} receptor over-expression into the nucleus accumbens to the ventral pallidum pathway decreases motivation for palatable food

Despite the possible bias caused by acute activation of the NAc $A_{2A}R$, this adenosine receptor subtype plays an important role in modulating MSNs activity in striatal areas through a function opposite to that of D_2R (Svenningsson et al., 1999) and, thus, contributes to the control of DA-mediated arousal and reward processes of appetitive behaviour (Volkow et al., 2011). In this sense, NAc DA modulates the performance of goal-directed behaviour and is involved in various aspects of instrumental learning and motivation (Ikemoto and Panksepp, 1996; Salamone and Correa, 2002; Salamone et al., 2007). These attributes are essential in understanding binge-eating disorder since this abnormal eating is a

chronic disturbance of the central nervous system that alters reward sensitivity, and patients with this pathology also show motivational dysfunction and increased impulsivity that may become a central deficit that disrupts their daily life (Wilson et al., 2019). Therefore, another focus of research in eating disorders would be the neuropathological mechanisms involved in the motivation-related behaviour that led to compulsive overeating, and the role of $A_{2A}R$ in modulating the NAc pathway output.

Motivated behaviour can be characterized by vigour, persistence, and high levels of work output. These activational aspects of motivated behaviour have enormous adaptive significance and are important in addiction research since most psychobiological models of drug addiction consider the motivational or reinforcing aspects of drugs to be the central drive for drug use. Over the past two decades, considerable researches have demonstrated that NAc DA is a critical component of the brain circuitry controlling effort-related behavioural processes (Salamone et al., 1991; Salamone and Correa, 2002; Phillips, Walton and Jhou, 2007). Depletions of NAc DA make animals susceptible to ratio requirements in operant schedules (Sokolowski and Salamone, 1998; Aberman and Salamone, 1999; Mingote et al., 2005). Furthermore, studies involving choice behaviour have shown that rats with NAc DA depletions or those treated with local intra-NAc injections of DA antagonists reallocate their behaviour away from food-reinforced tasks that have high response requirements by decreasing lever pressing and instead select less-effortful types of food-seeking behaviour (Koch, Schmid and Schnitzler, 2000; Nowend et al., 2001).

On the other hand, NAc $A_{2A}R$ activation resulted in similar behavioural effects described above. Indeed, bilateral infusions of CGS 21680 into the NAc decreased lever pressing for the preferred food but substantially increased consumption of the less preferred chow that required less effort to obtain (Font et al., 2008). The same local activation of NAc $A_{2A}R$ disrupted the performance of an instrumental task with high work demands (Mingote et al., 2008), similarly that occur after NAc DA depletions (Mingote et al., 2005). These behavioural results were investigated together with anatomical and neurochemical studies revealing that this effect was in part attributed to the stimulation of $A_{2A}R$ on NAc GABAergic neurons that project to the ventral pallidum (VP) (NAc indirect or "No Go" pathway) (Mingote et al., 2008). These findings added support to the activity of the ventral striatopallidal pathway as a key axis in the regulation of motivation and goal-directed action (Kelley, 2004; Phillips, Walton and Jhou, 2007; Salamone et al., 2007; Poyraz et al., 2016), and to the role of the adenosine $A_{2A}R$ in modulating the activity of this pathway. Accordingly, we proposed that the effects of NAc $A_{2A}R$ activation reported in the pharmacological approach on reducing the palatable food intake may be caused by regulating effort-related processes through activation of the indirect pathway in the NAc ("No Go" pathway) rather than affecting the hedonic value of the reward.

To further investigate this hypothesis, we developed a more accurate approach based on a combinatorial adeno-associated virus (AAV) that specifically upregulate the $A_{2A}R$ expression by targeting the NAc to VP pathway (**Figure 28**). First, we observed significant differences in increased food intake patterns in mice locally overexpressing $A_{2A}R$ compared to the AAV-CTRL group following repeated binge eating cycles. The relative

difference in food intake (kCal/g animal of standard food and cafeteria diet) from baseline conditions (before surgery) showed a progressive trend of increasing intake of highly palatable food (cafeteria diet) in the control injection group (AAV-CTRL-BE). However, this pattern of increased intake was significantly less in the A_{2A}R-overexpressing group (AAV-A2AR-BE) during the binge periods (the first 1.5 and 3 h) (**Figure 31c and f**). Interestingly, the differences observed between injection groups disappeared 24 h after the onset of the binge-like eating cycles (**Figure 31i**). These results suggested that the overexpression of A_{2A}R in the indirect pathway of the NAc caused a lower reactivity to novel environments and, therefore, less sensitivity to the rewarding effects of highly palatable food than control animals. Thus, we further investigated the seeking-related behaviour towards palatable food using a self-administration protocol in an operant model.

NAc DA is particularly important for tasks that require substantial effort to obtain the goal (Salamone et al., 2002, 2005, 2007). According to this, we assessed the A_{2A}R overexpression effect in regulating motivation-related processes for high palatable food (chocolate-flavour pellets) using an instrumental task with high effort requirements in an operant model, progressive ratio (PR) schedule. In our paradigm, mice subjected to A_{2A}R overexpression significantly reduced the breaking points in 5 h to obtain chocolate-flavour pellets as reinforcers in an operant self-administration protocol (**Figure 34a**), with no effect on a task with lower work demand (**Figure 33**). Both binge-like eating and standard group decreased the seeking-like behaviour for palatable food, indicating that the local overexpression of A_{2A}R in the NAc-VP pathway has a protective effect in

the motivation-related effort to obtain a reward even in mice exposed to intermittent free-choice access to highly caloric and palatable food.

Our results suggested that A_{2A}R-overexpression in the NAc indirect pathway ("No Go" pathway) may increase ventral striatopallidal transmission and modify motivational behaviours towards palatable food. Furthermore, A_{2A}Rs are co-expressed with D_2Rs in indirect pathway MSNs and are thought to oppose the D_2R function (Svenningsson et al., 1999). In agreement with this, recent studies reported opposite results on motivation behaviour following D_2R upregulation, revealing enhanced willingness to work for food in a PR task (Gallo et al., 2018). Altogether, these findings proposed the activity regulation of the NAc indirect pathway output as an interesting focus of research for overeating disturbances by modifying motivational disruptions.

Prolonged exposition to intermittent free-choice access impairs cognitive flexibility but do not increase vulnerability to food addiction

As indicated in the transcriptomic analysis discussed above, binge eating mice showed DEGs related to memory, learning and cognition. In addition, in human studies, obese binge eaters showed significantly higher game-changing and perseveration errors on cognitive flexibility and inhibitory control tests than obese without the binge-eating disorder. These results reveal important links between compulsive eating behaviour and executive function impairments. Thus, from a psychological and neurological perspective, we may speculate that repetitive binge eating of high caloric and palatable food alters the normal functioning of the brain's reward system, causing a pathological usurpation of the neural mechanisms of learning and memory that under normal circumstances

serve to shape survival behaviours related to the pursuit of rewards and the cues that predict them.

Based on this hypothesis, we assessed in the adenoviral gene delivery approach whether prolonged binge eating cycles modify the adaptive behavioural abilities and how $A_{2A}R$ overexpression might influence this behaviour. Using an operant self-administration approach, visual cues are associated with the availability of reinforcers, chocolate-flavoured pellets. The reversal test allows investigating the ability to change strategy to obtain the desired reinforcer again by calculating the hit ratio under a new condition. Therefore, cognitive inflexibility is measured through the mice's perseverance in seeking highly palatable food on the "old active lever" despite the absence of visual cues and the availability of the reinforcer. In our paradigm, mice exposed to repetitive binge-like eating cycles showed a significant reduction in the adaptive behavioural abilities, exhibiting impaired capacities to active lever pressing shift in a reversal test (**Figure 34f**). These results would support our transcriptomic findings in NAc D2(+) neurons discussed above, in which binge eating mice showed DEGs functionally associated with memory, cognition and learning. In addition, these findings suggested that the prolonged binge eating repetition of palatable and highly caloric food is a preclinical model that can "mimic" some of the cognitive impairments observed in humans (Duchesne et al., 2010; Svaldi, Brand and Tuschen-Caffier, 2010; Balodis et al., 2013). On the other hand, the mild trend increasing the adaptive behaviour in those mice belonging to the AAV-A2aR-BE group relative to AAV-CTRL-BE proposed that $A_{2A}R$ overexpression in the NAc-VP pathway may enhance cognitive flexibility in mice exposed to intermittent free-choice access to cafeteria diet.

In humans, the type of food ingested during binges differs among individuals and within a single individual. Therefore, binge eating appears to be characterized more by an abnormality in the amount of food consumed in a short period than by a craving for a specific nutrient (American Psychiatric Association, 2013). This behavioural feature would be one of the principal diagnostic criteria differentiating the binge-eating disorder from other high comorbid eating disturbances, like food addiction. Experiments in animals and humans showed that, for some people, a certain type of food enriched in sugar, fat and salt could trigger the same reward and pleasure centres of the brain (Mancino et al., 2015; Gearhardt, Corbin and Brownell, 2016; Domingo-Rodriguez et al., 2020). Like addictive drugs, people with food addiction lose control over their eating behaviour and find themselves spending excessive amounts of time involved with food and overeating. However, binge eating is frequently observed in people diagnosed with food addiction, and some of the addiction traits are described in binge eater persons. These important overlapping reveals that these two mental disorders share multiple transdiagnostic constructs and have high comorbidity (Davis et al., 2011; Gearhardt et al., 2012), making it difficult to establish the source of independence between them. In fact, some authors argue that the concept of food addiction may simply be a more acute and pathologically-dense form of binge-eating disorder (Davis, 2013; Gordon et al., 2018), while other opinions support that they may represent unique yet overlapping conditions (Gearhardt, White and Potenza, 2011). According to this controversial fact, we investigated whether prolonged exposure to repetitive binge eating may lead to increased vulnerability to food

addiction and if the $A_{2A}R$ overexpression in the NAc indirect pathway may result in a protective factor for food addiction-like behaviours.

For this purpose, the persistence and the inhibitory control were also investigated as phenotypic traits of food addiction with high-translational face validity to human addiction (Mancino et al., 2015; Domingo-Rodriguez et al., 2020). In our results, prolonged exposure to repetitive binge eating did not result in increased vulnerability to food addiction relative to the group of mice with standard *ad libitum* feeding conditions (AAV-CTRL-BE vs AAV-CTRL-STD), revealing the same percentage of vulnerability to food addiction for both diet groups (20%) (**Figure 34d**). On the other hand, the local overexpression of $A_{2A}R$ seemed to be a protective condition to develop a vulnerability to food addiction, resulting in 0% individual vulnerability in mice belonging to the AAV-A2aR-STD group and 15.38% in those from the AAV-A2aR-BE group. The results from diet groups (BE and STD) with the AAV-CTRL injection suggested that binge-eating disorder and food addiction are two eating disorders that can develop independently and, therefore, food addiction could not be necessarily the result of a more severe form of binge eating disorder.

One explanation for the presence of vulnerability to food addiction in the absence of binge eating may be related to the intrinsic "addictive" properties of certain types of food. While the palatability of sugar- and fat-rich foods may lead to addiction-related behaviours similar to those described in substance use disorder (Westwater, Fletcher and Ziauddeen, 2016; Gordon et al., 2018), the most common antecedent of binge eating is negative affect (American Psychiatric Association, 2013). In addition, other factors like interpersonal stressors, dietary restraint, negative feelings related to body weight, body shape, and boredom are

the principal triggers of compulsive overeating. Binge eating frequently appears to minimize or mitigate factors that precipitated the episode in the short term, but negative self-evaluation and dysphoria are often delayed consequences. Therefore, binge-eating disorder would be a compulsive-type behaviour associated with significant psychiatric comorbidity comparable to bulimia nervosa and anorexia nervosa, mainly triggered by negative emotional states or comorbidity with other mental disorders such as obsessive-compulsive disorder, anxiety and depression (American Psychiatric Association, 2013).

In sum, although food addiction is still a controversial concept (Gordon et al., 2018), understanding the relationship and differences between food addiction and binge-eating disorder may be informative in identifying the mechanisms underlying the development, maintenance, prevention, and treatment of problematic eating. The binge-eating disorder seems particularly linked to addiction given specific features of the disorder (e.g., compulsive eating, excess consumption despite adverse consequences, and diminished self-control over eating behaviours). However, food addiction may also be linked to other eating behaviours associated with non-BED-related obesity and implicate the hijack of the brain's reward system similar to the drug of abuse do it. The identification of the particularities among obesity, food addiction and compulsive overeating would be helpful to conceptualize eating behaviours, improve prevention strategies and identify potentially novel avenues for developing effective treatments.

Behavioural characterization of a mouse model of binge-like eating behaviour in PheComp cages

One reason for the limited progress in developing treatment strategies for abnormal eating behaviours is that physiological and neurological causes and consequences are still unclear and cannot readily be studied in human subjects. The main reason for this is the limited availability of preclinical models representing the individual diversity in a human population. In particular, the neuropathological mechanisms that identify binge-eating disorder and differentiate it from other highly comorbid eating disorders are especially difficult to study without a well-characterized preclinical model for binge-eating disorder. Therefore, improved versions of current preclinical models are needed to reproduce in animals the biology and symptoms observed in human patients.

Several mouse models of binge-like eating behaviour have been developed to investigate the neurobiological mechanisms of the binge-eating disorder. Most of them rely on food restriction or stress induction to identify mechanisms regulating excessive eating (Avena and Bocarsly, 2012). On the one hand, sucrose bingeing is a model of compulsive over-intake consisting of 12 h-period access to a 10% sucrose solution (or 25% glucose in earlier studies) and standard rodent chow, followed by 12 h of sucrose and chow deprivation, for about one month (Avena, 2007). Other rodent models reproduce binge-like eating in rats by combining caloric restriction and stressors like footshock (Hagan et al., 2002). The use of stressors and/or intermittent fasting allows reproducing the environmental conditions responsible for provoking, in some cases, the compulsive overeating reported in patients suffering from binge-eating disorder. However, these models have some disadvantages. For example,

overnight food deprivation causes hyperphagia, but at the same time, causes increased locomotor activity and significantly elevates corticosterone levels. Similarly occurs in the case of a combination of caloric restriction and stressors like footshock. Furthermore, the stability of behaviour in these models is often a limiting issue (Hagan and Moss, 1997; Dimitriou, Rice and Corwin, 2000; Davis et al., 2008).

A simpler model of binge-like eating behaviour was developed based on intermittent access to a high energy diet without food deprivation or the application of exogenous stressors (Czyzyk, Sahr and Statnick, 2010). The model described by Czyzyk et al. assesses whether the mice have consumed the food by binge eating by calculating the difference in weight between the initial and the final amount of food in a given period of time. Although this rodent model allows to reproduce binge eating episodes in a discrete period of time than controls under similar conditions and to induce a behaviour maintained over extended periods without using food deprivation, it also has some limitations. All mice subjected to intermittent access to palatable food show abnormal food consumption (binge eating episodes) in a homogeneous way. The high stability and reproducibility of this abnormal eating behaviour allow investigating the characteristics of compulsive overeating compared to other eating disorders, such as obesity and food addiction, as done in this Thesis. However, binge-eating disorder is an abnormal eating behaviour that involves compulsive-type behaviour towards food, and therefore other criteria besides the amount of food and energy ingested must be considered.

A current mouse model for compulsive-like feeding has developed analysis methods that assess different parameters representing aberrant

eating behaviours by using PheComp cages (Fructuoso et al., 2019). These cages use the weight transducer technology for measuring food and drink consumption, thus allowing a continuous signal and then a precise analysis of the animal meal pattern by the software. In this model, six parameters can be calculated to determine compulsivity towards palatable food and a high energy diet and explore the basic mechanisms underlying compulsive overeating. However, this protocol was designed to investigate compulsive-like eating following obesity development and was not focused on subpopulation distinction vulnerable for a particular disorder. Here we proposed an alternative mouse model that improves other binge-eating behaviour protocols due to the possibility of establishing intra- and inter-individual differences in a heterogeneous population of mice by identifying specific phenotypes that reproduce diagnostic criteria for the binge-eating disorder without obesity development or using fasting and exogenous stressors.

The use of PheComp cages in combination with intermittent access to high caloric and palatable food allowed to define in a detailed manner the rodent behaviour by measuring specific components of variability and analyzing the structural pattern longitudinally. Taking advantage of the previous protocol established for compulsive-like eating in obese-like fed mice using PheComp cages (Fructuoso et al., 2019), we developed comparison methods of basal and aberrant meal patterns analysis to investigate whether the model of limited access to cafeteria diet is sufficient to recapitulate the features of clinical diagnostic criteria used in psychiatric manuals (American Psychiatric Association, 2013). According to the protocol previously published by Fructuoso et al., we first investigated six criteria that were representative of an aberrant feeding

pattern during free-choice conditions compared to baseline: total food intake (amount of food consumed gr food/gr animal), total energy intake (amount of energy, kCal food/gr animal), number of meals, eating rate (mg food/sec), the average duration of meals and satiety rate (sec/kCal food). These six criteria can be measured objectively and with a high level of individual detail in a heterogeneous population of mice.

Then, we aimed to identify extreme subpopulations of mice exhibiting aberrant behavioural traits similar to the diagnostic criteria for binge-eating disorder. To do this, we first established the minimum period necessary to elicit significant changes in the feeding pattern. Thus, we compared behavioural differences between early and late periods of intermittent access to highly palatable food protocol to determine whether prolonged exposure to binge eating episodes can lead to more exacerbated and aberrant eating behaviours. When mice were given a choice between standard food and a highly palatable chocolate-containing diet (cafeteria diet), they rapidly developed a lack of flexibility in food taking, reflected by a rejection of standard food when cafeteria diet is available (**Figure 38b**). In comparison with basal conditions (*ad libitum* access to standard food), mice modified their meal pattern increasing the number of meals, the amount of food and energy consumed, together with a significant reduction in the average duration of meals. This overconsumption is observed from the beginning of the protocol (early period, BC2) (**Figure 38b**). However, the modifications of eating patterns became more severe after six weeks of limited access to highly caloric and palatable food (final period, BC7) (**Figure 39a**), leading to behavioural changes even in the absence of the palatable food and in the normal pattern of circadian organization. Following seven cycles of binge

eating episodes, mice showed an increased preference for the feeder that previously contained the palatable food (**Figure 40a**) with maintained persistence throughout the active phase (12 h) (**Figure 39b**). These results would be in accordance with the previously cognitive flexibility impairment reported in this Thesis using an instrumental task in an operant-behaviour assay, indicating that repetitive binge eating can lead to the inability to adapt food-seeking behaviour. This fact has been previously described in limited access conditions (Heyne et al., 2009; Espinosa-Carrasco et al., 2018). However, in our paradigm, the exposition to previous obesogenic conditions was unnecessary to develop similar behavioural disturbances associated with compulsive-like eating.

Additionally, this alternative mouse model also allowed to investigate other phenotypic traits that may be disrupted following repetitive binge eating. For example, intermittent exposition to free-choice access to cafeteria diet also disrupted the circadian feeding organization compared to basal conditions (**Figure 38**). When mice are in free-choice conditions, they lose circadian feeding rhythmicity with interindividual differences. These results were similar to those previously described for obese-like fed mice (Espinosa-Carrasco et al., 2018) and in agreement with studies that reported a disruption of circadian molecular and behavioural rhythm after several weeks (Kohsaka et al., 2007; Hatori et al., 2012), or immediately after high-fat exposure (Pendergast et al., 2013). However, in our model, the behavioural alterations were maintained following seven weeks (**Figure 39a**), even in the absence of cafeteria diet (**Figure 39b**). These results suggested that changes triggered by repetitive binge eating were not transient and maintained over extender periods of time, contrary to the previously reported for *ad*

libitum access to cafeteria diet, where circadian rhythm was only disrupted during the first three weeks (Espinosa-Carrasco et al., 2018). In addition, the circadian disruption reported using metabolic boxes proposed comorbidity between binge-eating disorder and other disorders eating such as night-eating syndrome (NES) (Allison et al., 2005; Milano et al., 2012), which core feature is the delayed circadian shift of eating (O'Reardon et al., 2004), together with insomnia and distress (Stunkard, Grace and Wolff, 1955).

Then, as described above, the intermittent free-choice access to cafeteria diet rapidly modifies the meal pattern, altering homeostatic control of eating. This behavioural modification is triggered by a novel environment effect and the high palatability of the food. However, after six weeks of intermittent free-choice conditions, mice showed inter-individual differences in energy and food intake and other behavioural parameters (food-seeking behaviour and circadian rhythmicity) that suggested an altered feeding pattern beyond a one-off effect of the intermittent presence of a reinforcer. According to these results, we established that six weeks of intermittent free-choice access to the cafeteria diet was sufficient to generate significant modifications in meal patterns to distinguish different subpopulations of mice vulnerable to binge-eating disorder in a heterogeneous population.

A binge eating episode is characterized by both the ingestion of a large amount of food in a discrete period of time (Criterion A1) and a sense of lack of control over eating during the episode (Criterion A2) (American Psychiatric Association, 2013). Therefore, the first 3 h from the presentation of the cafeteria diet was established as the discrete-time period for a binge eating episode, and the eating rate (mg of food/sec)

recorded during such period was considered as a quantifiable and objective sign of loss of self-control over food intake. Five of the six quantifiable criteria described above (eating rate, average duration of meals, numbers of meals, total food and energy intake) were then considered to be hallmarks of binge-eating disorder (see **Figure 41**). First, the eating rate showed an increasing trend in the late period compared to the early period. Mice in the second cycle of binge eating showed a low interindividual variability with very similar eating rate data. However, after six weeks of intermittent free-choice access to the cafeteria diet, mice revealed high inter-individual variability, suggesting an interesting differentiation of extreme subpopulations with mice more vulnerable to loss of self-control over palatable food intake (**Figure 42a**). The cafeteria diet's total energy and food consumption significantly increased in the late period compared with the early period, without changes in the standard food consumption (**Figure 42d and e**). In addition, the number of meals, that is, the time elapsed until re-feeding, were shorter in the cafeteria diet feeder, indicating a low satiety potential (**Figure 42c**)**.** These results revealed that the repetitive exposition to binge eating episodes lead to intake of large amounts of food despite the high energy amount consumed, but with important interindividual differences. Based on this individual differentiation, we concluded that mice subjected to intermittent free-choice access during a prolonged period could be divided into two extreme subpopulations, vulnerable (22.27%) or resistant (72.73%) to binge-eating disorder, according to the total criteria achieved (**Figure 42f**).

Altogether, the prolonged exposition to intermittent free-choice access to cafeteria diet led to abnormal eating behaviour even in the

absence of palatable food. In addition, the increase in the amount of food and energy intake are helpful criteria to determine compulsive overeating but cannot be considered the unique criteria to determine binge-eating disorder. Thus, well-characterized animal models are needed to advance our understanding of binge-eating disorder to delineate better the similarities and differences with other abnormal eating behaviours. Furthermore, the model and analyses presented here provided an excellent tool to explore the aetiological or susceptible genetic factors in rodents without comparing them to other eating disorders (such as obesity and food addiction). Including genetic models (e.g. transgenic or knockout animals) of obesity-prone and obesity-resistant mouse strains would be possible to decipher whether genetic factors associated with obesity increase or decrease the vulnerability to binge-eating disorder. The extreme population identification also allows a better understanding of the aetiological contribution of specific transmission pathways or receptors comparing vulnerable and resistant subpopulations. Additionally, hormonal studies can also be carried out to investigate vulnerable features concerning sex. Although the gender ratio prevalence is less skewed in binge-eating disorder than in other eating disorders such as bulimia nervosa (2:1 vs 5:1, women:men) (Hudson et al., 2007), the identification of extreme population may be helpful to report more translational preclinical results about similarities and differences between men and women in a human population.

In conclusion, the present Doctoral Thesis has provided new insights into understanding the neurobiological mechanisms involved in the causation of binge-eating disorder, which could help to identify novel pharmacological approaches to address this pathology. Furthermore, the

comparative assessment of compulsive overeating and other abnormal eating behaviours, such as obesity and food addiction, has improved the understanding of the biology underlying binge-eating disorder and thus allowed the development of a preclinical mouse model that recapitulates most of the symptoms described in the diagnostic criteria for binge-eating disorder in humans.

CONCLUSIONS

The main conclusions of the work presented in this Thesis can be summarized as follows:

1. Alterations in the transcriptomic profile of NAc D2(+) neurons demonstrate similarities across different pathological disorders, such as drug addiction and binge-eating disorder, which provides insights into dimensional mechanistic similarities that might cut across seemingly differing behaviours.
2. The conditional binge-like eating episodes by intermittent free-choice access to highly caloric and palatable food can produce changes in DA signalling that differ from those with prolonged exposure to *ad libitum* free-choice access.
3. Transcriptomic analysis reveals an increase in the *Cck* gene mRNA in the NAc D2(+) neurons of binge-like eating mice compared to obese-like fed and standard mice that could underlie a CCK action in regulating food intake by reward-related behaviour.
4. The repetitive binge eating episodes trigger molecular signals to decrease 2-AG levels to restore homeostatic feeding by increasing MGLL synthesis.
5. Endogenous depletion of 2-AG levels in the NAc is sufficient to decrease the hedonic impact of palatable food by reducing the cafeteria diet consumption and increasing the less-preferred food intake during palatable food availability.
6. Adenosine A_{2A} receptor over-expression into the nucleus accumbens to the ventral pallidum pathway decreases motivation for palatable food.

7. Prolonged exposition to repetitive binge eating episodes impairs cognitive flexibility.
8. Prolonged exposure to repetitive binge eating does not result in increased vulnerability to food addiction, while local overexpression of $A_{2A}R$ in the NAc indirect pathway results in a protective factor to develop vulnerability to food addiction.
9. Six weeks of intermittent free-choice access to cafeteria diet is sufficient to generate significant modifications in meal pattern, altering homeostatic control of eating during the first 3 h from the cafeteria diet presentation.
10. Our results provide five quantifiable criteria as hallmarks of binge-eating disorder to distinguish different subpopulations of mice as vulnerable or resistant to binge-eating disorder in a heterogeneous population of mice.
11. The comparative assessment of compulsive overeating and other abnormal eating behaviours has improved the understanding of the neurobiology underlying binge-eating disorder and allowed the development of a preclinical mouse model that recapitulates most of the symptoms described in the binge-eating disorder's diagnostic criteria.

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ANNEX

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ARTICLE OPEN

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Reduced cue-induced reinstatement of cocaine-seeking behavior in *Plcb1* +/− mice

Judit Cabana-Domínguez[1,2,3,4,7], Elena Martín-García[5,6,7], Ana Gallego-Roman[5], Rafael Maldonado[5,6,8], Noèlia Fernàndez-Castillo[1,2,3,4,8]✉ and Bru Cormand[1,2,3,4,8]✉

Cocaine addiction causes serious health problems, and no effective treatment is available yet. We previously identified a genetic risk variant for cocaine addiction in the *PLCB1* gene and found this gene upregulated in postmortem brains of cocaine abusers and in human dopaminergic neuron-like cells after an acute cocaine exposure. Here, we functionally tested the contribution of the *PLCB1* gene to cocaine addictive properties using *Plcb1*+/− mice. First, we performed a general phenotypic characterization and found that *Plcb1*+/− mice showed normal behavior, although they had increased anxiety and impaired short-term memory. Subsequently, mice were trained for operant conditioning, self-administered cocaine for 10 days, and were tested for cocaine motivation. After extinction, we found a reduction in the cue-induced reinstatement of cocaine-seeking behavior in *Plcb1*+/− mice. After reinstatement, we identified transcriptomic alterations in the medial prefrontal cortex of *Plcb1*+/− mice, mostly related to pathways relevant to addiction like the dopaminergic synapse and long-term potentiation. To conclude, we found that heterozygous deletion of the *Plcb1* gene decreases cue-induced reinstatement of cocaine-seeking, pointing at PLCB1 as a possible therapeutic target for preventing relapse and treating cocaine addiction.

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INTRODUCTION

Cocaine is the most used psychostimulant illicit drug worldwide [1], causing severe health problems that include the development of cocaine addiction in around 15–16% of cocaine users [2]. Cocaine addiction is a complex psychiatric disorder that results from the interaction of genetic, epigenetic, and environmental risk factors [3]. The heritability of cocaine addiction is one of the highest among psychiatric disorders, estimated around 65% for women [4] and 79% for men [5]. However, the genetic factors and mechanisms that underlie the transition from drug use to addiction and its establishment remain unknown.

In a previous study, we identified a single nucleotide polymorphism in the *PLCB1* gene associated with drug dependence (rs1047383), and especially with a subgroup of cocaine-addicted patients, that was replicated in an independent clinical sample [6]. Also, genetic variants in this gene were found nominally associated with an illegal substance and cocaine addiction in two GWAS performed in European-American samples [7]. On the other hand, we found that cocaine increased the expression of *PLCB1* both in human dopaminergic neuron-like cells (differentiated SH-SY5Y cells) after acute cocaine exposure and in postmortem samples of the nucleus accumbens (NAc) of cocaine abusers [6]. Interestingly, this gene was also found over-expressed in the same brain region in mice after cocaine administration for 7 days and also during withdrawal [8]. All this evidence suggest that *PLCB1* may play a role in cocaine addiction.

The *PLCB1* gene encodes phospholipase C beta 1, and it is highly expressed in the brain, mainly in the frontal cortex, basal ganglia (caudate, putamen, and NAc) and hippocampus (HPC). These brain regions are crucial for drug reward and the formation of drug-context associations, both contributing to the development and maintenance of addiction [9–14]. Several neurotransmitters activate this protein, including dopamine through DRD1 and DRD2 [15, 16], serotonin by 5-HT2A and 2C receptors [17, 18] and glutamate by mGluR1 [19, 20]. Thus, PLCB1 might be a point of convergence of neurotransmitter systems that play an essential role in the development of addiction (recently reviewed [21, 22]). The activation of PLCB1 produces the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP3), responsible for intracellular signal transduction. Alterations in PLCB1-mediated signaling in the brain have been associated with other neuropsychiatric disorders such as epilepsy, schizophrenia, and bipolar disorder [23].

Here we studied the contribution of the *PLCB1* gene to cocaine addiction and dissected its participation in the different aspects of the addictive process. We used heterozygous knockout (KO) mice (*Plcb1*+/−), as the homozygous KO (*Plcb1*−/−) showed seizure

[1]Department de Genètica, Microbiologia i Estadística, Facultat de Biologia, Universitat de Barcelona, Barcelona, Catalonia, Spain. [2]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain. [3]Institut de Biomedicina de la Universitat de Barcelona (IBUB), Barcelona, Catalonia, Spain. [4]Institut de Recerca Sant Joan de Déu (IR-SJD), Barcelona, Catalonia, Spain. [5]Laboratory of Neuropharmacology-Neurophar, Department of Experimental and Health Sciences, Universitat Pompeu Fabra (UPF), Barcelona, Catalonia, Spain. [6]Hospital del Mar Medical Research Institute (IMIM), Barcelona, Catalonia, Spain. [7]These authors contributed equally: Judit Cabana-Domínguez, Elena Martín-García. [8]These authors jointly supervised this work: Rafael Maldonado, Noèlia Fernàndez-Castillo, Bru Cormand. ✉email: noefernandez@ub.edu; bcormand@ub.edu

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attacks and low viability after birth [24]. In addition, the use of a constitutive heterozygous KO mouse model allowed us to assess *Plcb1* haploinsufficiency during neurodevelopment in mice similarly to humans, where inherited genetic risk or protective variants can modulate the susceptibility to addiction [6]. First, we performed a general phenotypic characterization using a battery of behavioral tests, including memory, anxiety, locomotor activity, coordination, food and water intake, and sucrose preference. Then, we evaluated cocaine operant self-administration, extinction, and cue-induced reinstatement. Finally, we studied transcriptional alterations to further understand the molecular mechanisms involved.

METHODS

Animals

Male mice, 8 weeks old, were housed individually at temperature- and humidity-controlled laboratory conditions (21 ± 1 °C, 55 ± 10%) maintained with food and water ad libitum. Mice were tested during the dark phase of a reverse light cycle (lights off at 8.00 h and on at 20.00 h). *Plcb1*+/− mice in a C57BL/6J background and their wild-type (WT) littermates were used [24]. All experimental protocols were performed in accordance with the guidelines of the European Communities Council Directive 2010/63/EU and approved by the local ethics committee (Comitè Ètic d'Experimentació Animal-Parc de Recerca Biomèdica de Barcelona, CEEA-PRBB, agreement N °9213). In agreement, maximal efforts were made to reduce the suffering and the number of mice used.

Western blot against Plcb1

Medial prefrontal cortex (mPFC) and HPC from mice not exposed to cocaine ($n = 4$ WT and 4 *Plcb1*+/− mice) were dissected. Protein lysates and western blot experiments were performed as previously described [25] using a primary antibody against Plcb1 (sc-5291, Santa Cruz Biotechnology, Texas, USA) diluted 1:500, and a secondary anti-mouse IgG antibody (A0545, Sigma-Aldrich, UK) diluted 1:10,000. For normalization, α-tubulin levels were measured with an anti-α-tubulin antibody (T5168, Sigma-Aldrich, UK; diluted 1:2.000).

Behavioral tests for phenotype characterization

A group of 25 WT and 12 *Plcb1*+/− mice underwent different behavioral tests for phenotype characterization for 10 days, as described in Fig. 1. Mice were first tested for short-term memory of a novel object recognition task on a V-maze apparatus. After this test, locomotor activity was evaluated in actimetry boxes. Anxiety-like behavior was then assessed by using the elevated plus maze test, and finally motor coordination was evaluated using the rota rod test. In addition, six animals per genotype were placed in experimental PHECOMP boxes (Panlab and Harvard Apparatus) to control several consummatory and locomotor parameters. Every day position bottles was exchanged, and the consumption was measured after a 24 h interval.

Operant conditioning maintained by cocaine

Cocaine self-administration experiments were performed in the same animals as previously described [26, 27], after the behavioral tests for phenotype characterization. Each 2 h daily self-administration session started with a priming injection of the drug. Cocaine was intravenously infused in 23.5 µl over 2 s (0.5 mg/kg per injection). Cue light, located above the active hole, was paired with the delivery of the reinforcer. Mice (WT $n = 36$; *Plcb1*+/− $n = 26$) were trained under a fixed ratio 1 schedule of reinforcement (FR1; one nose-poke lead to the delivery of one dose of cocaine) over five consecutive daily sessions and under a fixed ratio 3 (FR3) over five consecutive daily sessions. Control mice trained with saline were included for both genotypes (WT $n = 6$; *Plcb1*+/− $n = 6$). After the ten FR sessions, animals were tested in a progressive ratio schedule of 4 h where the response requirement to earn the cocaine escalated according to the following series: 1–2–3–5–12–18–27–40–60–90–135–200–300–450–675–1000. Then, we proceed with the extinction phase, 2 h daily sessions following the same experimental conditions than the cocaine self-administration sessions except that cocaine was not available, and cue-light was not presented. Finally, mice that accomplished extinction criterion were tested in the cue-induced reinstatement during a 2 h session, to evaluate the reinstatement of cocaine-seeking behavior.

After the cue-induced reinstatement, animals were euthanized by decapitation, brains were quickly removed, and the mPFC and HPC were dissected. Brain tissues were then frozen by immersion in 2-methylbutane surrounded by dry ice and stored at −80 °C for later RNA isolation and transcriptomic analyses.

RNA extraction and RNA sequencing

Total RNA of 12 WT and 11 *Plcb1*+/− mice from mPFC and HPC were isolated using the RNeasy Lipid Tissue Mini Kit (Qiagen Düsseldorf, Germany) according to the manufacturer's protocol. RNA samples were grouped in four pools consisting of three mice per pool for each experimental group. Pools were homogeneous in the average number of nose pokes in the cue-induced reinstatement and representative from the whole sample. Furthermore, no significant differences were observed in the mean number of cocaine infusions in 2 h sessions among pools with an average of 28.5 ± 0.64 infusions in Plcb1+/− mutants and in 26.48 ± 1.23 in WT, suggesting that any transcriptomic change found would be related to the addiction phenotype and not to the concentration of cocaine in the brain in the previous phase of acquisition (t-test = 1.42, n.s.).

RNA sequencing (RNAseq) was performed by the Centre de Regulació Genòmica (CRG, Barcelona, Spain). Libraries were prepared using the TruSeq Stranded mRNA Sample Prep Kit_v2 (Illumina, San Diego, CA, USA) according to the manufacturer's protocol and sequenced 2 × 75 on Illumina's HiSeq3000 system for both mPFC and HPC. The Bioinformatics service of CRG carried out the analysis of RNAseq. Briefly, FastQC v0.11.5 [28] was used to inspect the reads quality and CutAdapt 1.7.1 [29] to clean the data of adapters and low-quality reads. Then, reads were mapped to the *Mus musculus* genome of reference (GRCm38/mm10) with STAR 2.5.3a [30], and the differential expression analysis was done by DESeq2 [31] to compare WT and *Plcb1*+/−. Corrections for multiple testing were applied by adjusting the p values with a 5% False Discovery Rate.

Functional annotation of RNAseq results

We performed a functional group enrichment of differentially expressed genes (DEGs) in mPFC using the DAVID Annotation Tool (http://david.abcc.ncifcrf.gov) [32] considering GO (Gene Ontology) biological processes and KEGG pathways (Kyoto Encyclopedia of Genes and Genomes). Then, we searched for over-represented transcription factor-binding sites (TFBS) using the information of MsigDB (https://www.gsea-msigdb.org/gsea/msigdb) integrated on WebGestalt2019 (http://www.webgestalt.org/) [33], and the default parameters, applying the weighted set cover method to reduce redundancy. In both analyses, the Benjamin–Hochberg procedure was performed for multiple testing. Finally, we investigated the existence of gene networks with Ingenuity Pathway Analysis 8.8 software (IPA, http://www.ingenuity.com/products/ipa; Ingenuity Systems, Redwood City, CA, USA) [34] after selecting genes with fold-change (FC) > |1.2 | .

Statistical analysis

Mice were randomly allocated in their experimental groups and experiments were performed under blind conditions. Three-way ANOVA with repeated measures was used to test the evolution over sessions or days. Sessions or days were used as within-subject factors and genotype (*Plcb1*+/− or WT) and drug (cocaine or saline) were used as between-subjects factors. Post-hoc analyses (Newman–Keuls) were performed when required. Comparisons between two groups were analyzed by Student t test or U-Mann–Whitney depending on the distribution defined by the Kolmogorov–Smirnov normality test and the sample size. The chi-square analyses were performed to compare the percentage of mice that acquired operant learning criteria in the different experimental groups. Results are expressed as mean ± SEM or individual values with the median and the interquartile range specified in the figure legend. Differences were considered significant at $P < 0.05$. The sample size was calculated based on power analysis using two-sample Student t test, achieving a power between 80 and 90%. The statistical analyses were performed using the Statistical Package for Social Science tool SPSS®25.0 (SPSS Inc, Chicago, USA).

See supplementary information for more details of genotyping of transgenic mice, drugs, behavioral tests of phenotype characterization, operant conditioning maintained by cocaine, RNA extraction and sequencing.

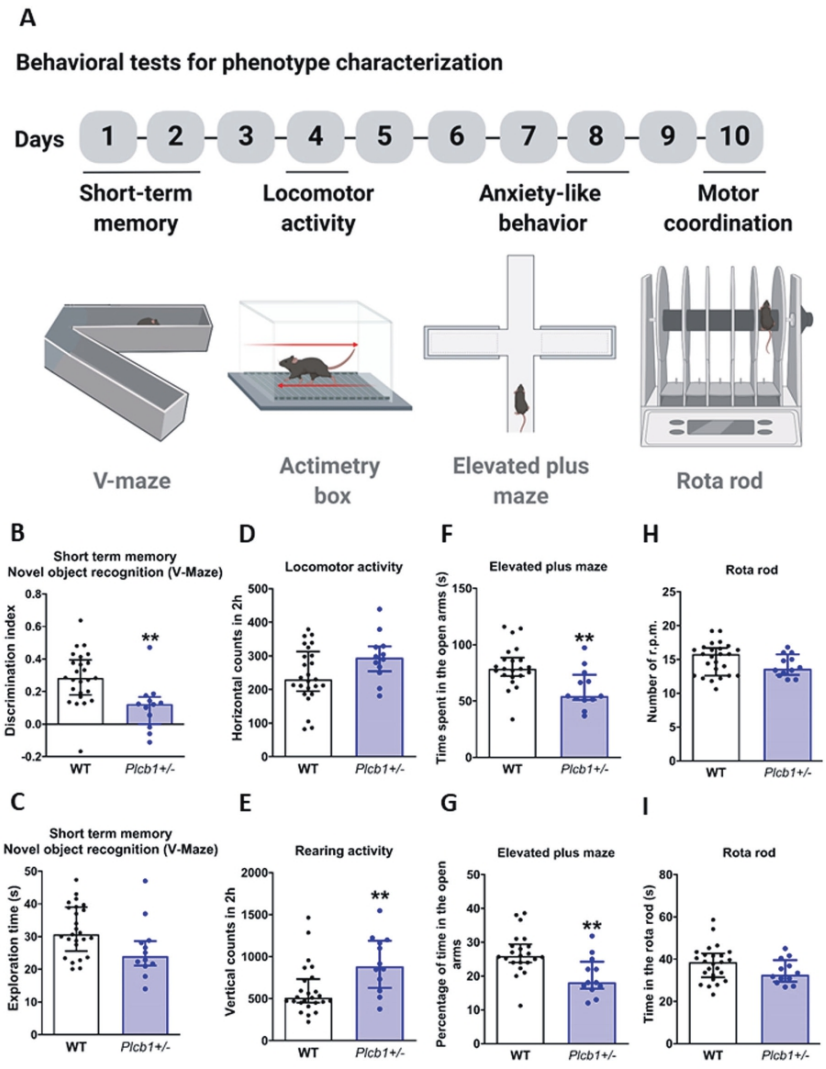

Fig. 1 Behavioral tests for phenotype characterization. A Timeline of the experimental sequence. **B**, **C** Short-term memory measured in the V-maze for novel object recognition task, (**B**) discrimination index (*t*-test, $^{**}P < 0.01$ vs WT) and (**C**) exploration. **D** Locomotor and (**E**) rearing activity measured in actimetry boxes (*U*-Mann–Whitney, $^{**}P < 0.01$ vs WT). **F** Anxiety-like behavior measured by the time spent in the open arms (*t*-test, $^{**}P < 0.01$ vs WT) and (**G**) the percentage of time in the open arms in the elevated plus maze (*t*-test, $^{**}P < 0.01$ vs WT). Motor coordination measured in the rota rod test by the (**H**) number of r.p.m. that the mice performed in the average trials or the (**I**) mean time they remain in the apparatus. All data are expressed in median and interquartile range and individual data are shown (WT $n = 25$; *Plcb1*+/− $n = 12$).

RESULTS

In the present work, we aimed to assess the contribution of the *PLCB1* gene in the different stages of cocaine addiction using heterozygous KO mice (*Plcb1*+/−). To do so, we performed a general phenotypic characterization and, then we evaluated cocaine operant self-administration, extinction, and cue-induced

reinstatement in those animals. Also, we studied transcriptional changes in mPFC and HPC to understand the mechanisms involved.

Behavioral tests for phenotype characterization

First, we confirmed by western blot that protein levels of Plcb1 were reduced in the brain regions of interest, about 22%, on the *Plcb1*+/− mice compared to WT in mPFC and HPC ($P = 0.013$ and $P = 0.018$, respectively; Supplementary Fig. S1). Importantly, this reduction was observed in Plcb1a isoform but not in Plcb1b, both in mPFC and HPC, and there seems to be a slight compensatory mechanism.

Then we evaluated the effects of the heterozygous deletion of *Plcb1* on general behavioral responses, including locomotor, cognitive and emotional responses, food, and water intake. *Plcb1*+/− mice showed a lower discrimination index in the novel object recognition compared to WT (*t*-test = 3.21, $P < 0.01$, Fig. 1B), suggesting an impairment in short-term memory. This difference was not influenced by exploration time, as this variable was equal between genotypes (Fig. 1C). Furthermore, no differences in locomotor activity were reported between genotypes, discarding an involvement of the *Plcb1* heterozygous deletion in locomotion (Fig. 1D). Mutant mice showed increased exploratory activity with a higher number of rearings than the WT mice (*U*-Mann–Whitney = 67.5, $P < 0.01$, Fig. 1E). An anxiogenic profile was revealed in the elevated plus maze in mutants, as shown by the reduced time spent in the open arms (*t*-test = 3.02, $P < 0.001$, Fig. 1F) and the percentage of time (*t*-test = 3.16, $P < 0.001$, Fig. 1G). Finally, the rota rod test revealed that the heterozygous deletion of *Plcb1* did not affect motor coordination (Fig. 1H, I).

Several consummatory and locomotor parameters were long-term monitored in the PheComp boxes (Supplementary Fig. 2A–H, $n = 6$ per genotype). No differences between genotypes were revealed during the whole experimental period in body weight, food, and water intake, levels of sucrose preference, stereotyped movements, horizontal and vertical locomotor activity, indicating no altered behavior in the *Plcb1*+/− mice.

Operant conditioning maintained by cocaine

Then, we investigated the effects of the heterozygous deletion of *Plcb1* in cocaine behavioral responses related to its addictive properties. For this purpose, *Plcb1*+/− mice and their WT littermates were trained for cocaine operant self-administration (0.5 mg/kg/infusion) during FR1 and FR3, progressive ratio, extinction and cue-induced reinstatement (Fig. 2A). Control mice trained with saline were included for both genotypes. Results showed that the percentage of mice reaching the criteria of operant conditioning was 100% for both genotypes trained with cocaine and 33% for mice trained with saline: [*Chi-square* test = 9.50; $P < 0.01$, *Plcb1*+/− cocaine vs *Plcb1*+/− saline] and [*Chi-square* test = 14.00; $P < 0.001$, WT cocaine vs WT saline], as expected.

The primary reinforcing effects and the motivation for cocaine were similar in both genotypes (Fig. 2B, C). During FR1, both genotypes similarly increased the number of cocaine infusions across sessions, whereas mice trained with saline remained steady (repeated measures ANOVA, interaction between genotype × drug × sessions, $P < 0.05$, Fig. 2B and Supplementary Table S1). Thus, the evolution of operant responding was different in mice trained with cocaine and saline, with an increased responding over nearly all sessions in cocaine-trained mice. This enhancement in cocaine responding was more pronounced in mutants than WT mice. Indeed, WT mice showed a decrease in cocaine intake in the second session. The maximum number of infusions was reached on session 5 in both genotypes and was slightly superior in *Plcb1*+/− mutants (36.35 ± 1.47) than in WT (29.61 ± 2.23) but post-hoc analyses demonstrated that this difference in cocaine intake was not significant (Fig. 2B). Similar findings were observed for the total number of nose-pokes in which operant responding increased in both genotypes with cocaine but remained stable with saline (Supplementary Fig. S3A). When the effort to obtain one dose of cocaine increased to FR3, the number of infusions was stable across sessions in WT and *Plcb1*+/− mice. Similarly, operant responding was higher for all groups than in FR1 with stable higher levels of responding (Supplementary Fig. S2A) and a higher number of infusions (repeated measures ANOVA, the main effect of the drug, $P < 0.001$, Fig. 2B) in mice trained with cocaine than with saline, independently of the genotype.

Motivation for cocaine was evaluated in a progressive ratio schedule, and no significant differences were obtained between genotypes (Fig. 2C). The levels of extinction of the operant behavior were similar between genotypes and decreased progressively across sessions (repeated measures ANOVA, interaction genotype × sessions, $P < 0.001$, Fig. 2D). The percentage of mice reaching cocaine-seeking extinction criteria was similar in *Plcb1*+/− (73%) and WT (78%) mice.

Importantly, *Plcb1*+/− mice showed significantly reduced cue-induced reinstatement of cocaine-seeking compared to WT mice (*U*-Mann–Whitney, $P < 0.05$, Fig. 2E), with 27.59% less active nose-pokes compared to WT mice trained with cocaine. Furthermore, mice trained with saline from both genotypes exhibited 55.84% reduction of active nose-pokes than WT mice trained with cocaine and 39.20% less than mutants trained with cocaine. No significant differences were obtained between genotypes in inactive nose-pokes during operant conditioning maintained by cocaine nor during extinction (Supplementary Fig. S3B–C). Both genotypes trained with cocaine acquired the reinstatement criterion (double nose pokes in the active hole than the number of nose pokes during the 3 consecutive days when the mice acquired the extinction criteria) showing their capability to maintain this conditioning learning task. These data showed that *Plcb1*+/− resulted in a phenotype of decreased of cue-induced reinstatement with reduced cocaine-seeking (Fig. 2E).

Brain transcriptomic analysis after the reinstatement of cocaine-seeking behavior

To further understand the role of PLCB1 in the molecular mechanisms involved in this cocaine relapse-related phenotype, we analyzed the transcriptomic profiles of mPFC and HPC immediately after the reinstatement of cocaine-seeking behavior in WT and *Plcb1*+/− mice trained with cocaine. We identified 2115 protein-coding genes differentially expressed (DEGs) in mPFC (1231 downregulated and 943 upregulated) and only 12 in HPC, when comparing *Plcb1*+/− and WT animals (Supplementary Tables S2–3). In accordance, principal component analysis (PCA) and heatmap plots revealed that individuals with the same genotype plotted together only in mPFC, but not in HPC (Supplementary Fig. S4). This suggested a more prominent role of mPFC, so further studies were carried out only with DEGs of this brain area. Analysis of functional group over-representation identified several processes previously related to cocaine addiction, including dopaminergic synapse, learning, long-term potentiation (LTP), neurotransmitter secretion, and axon guidance, as well as other relevant signaling pathways such as MAPK, mTOR, and neurotrophin (Fig. 3A, B and Supplementary Tables S3–5). We focused on the dopaminergic synapse pathway (enriched in the mPFCs DEGs; $P_{Adj} < 0.05$), in which phospholipase c (such as Plcb1) is directly participating in signal transmission (Fig. 4). Interestingly, many genes coding for proteins in this pathway are differentially expressed in *Plcb1*+/− mice after cue-induced reinstatement (Fig. 4 and Supplementary Table S6). Furthermore, we found that several TFBS were over-represented in the DEGs of mPFC, including YY1, MYOD, NRF1, ERR1, FREAC2, NFY and E4F1 (complete list of TFBS in Supplementary Table S7). Then, we filtered the DEGs on mPFC based on fold-change (FC > | 1.2 |) and

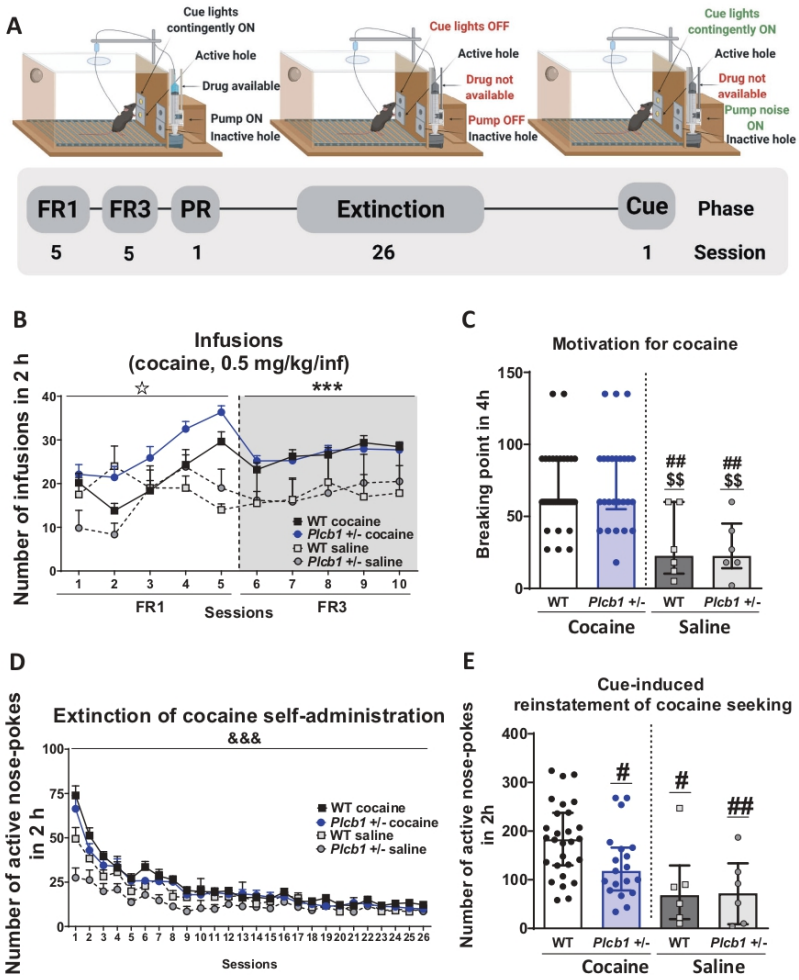

Fig. 2 Operant conditioning maintained by cocaine in *Plcb1*+/−, wild-type (WT) and corresponding control saline mice. A Timeline of the experimental sequence. **B** The number of cocaine infusions (0.5 mg/kg/infusion) in both genotypes increased progressively across sessions during FR1 (repeated measures ANOVA, interaction between genotype × drug × sessions, $^{\star}P < 0.05$) and remained stable during FR3 and higher in mice trained with cocaine (repeated measures ANOVA, main effect of drug, $^{***}P < 0.001$). The maximum number of infusions was reached on session 5 in both genotypes and was slightly superior in *Plcb1*+/− (36.35 ± 1.47) than in WT (29.61 ± 2.23) but post-hoc analyses demonstrated that this difference was not significant. **C** The motivation for cocaine was equivalent in both genotypes trained with cocaine but higher than those trained with saline (individual data with interquartile range, *U*-Mann–Whitney, $^{\#\#}P < 0.01$ vs WT cocaine, $^{\$\$}P < 0.01$ vs *Plcb1*+/− cocaine). **D** Both genotypes also showed similar levels of extinction that decreased during sessions, but the curve was more pronounced in WT mice than in mutants (repeated measures ANOVA, interaction genotype × sessions, $^{\&\&\&}P < 0.001$). **E** Decreased cue-induced reinstatement of cocaine-seeking in *Plcb*+/− mice was obtained compared to WT (individual data with interquartile range, *U*-Mann–Whitney, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs WT cocaine). All data are expressed in mean ± SEM when sessions are represented or median and interquartile range when individual data are shown (WT cocaine $n = 28$-36; *Plcb1*+/− cocaine $n = 19$–26; saline $n = 6$ per genotype). Statistical details are included in Supplementary Table S1.

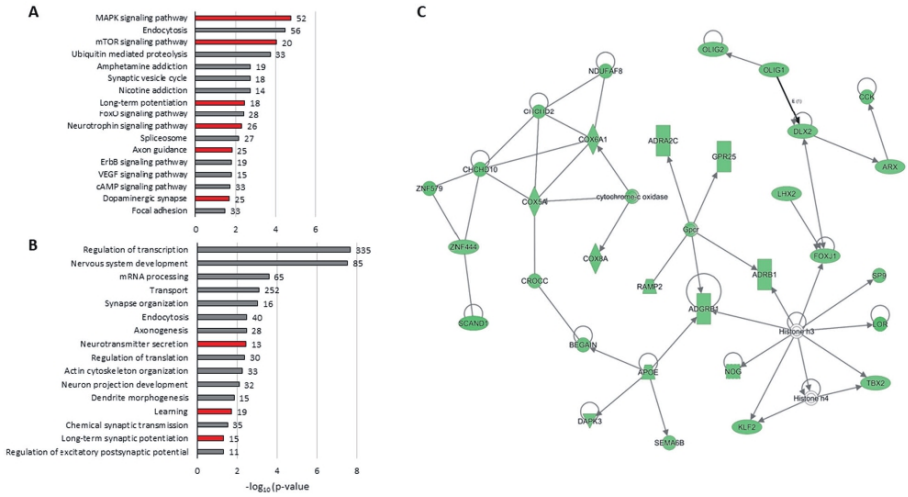

Fig. 3 Gene expression changes in mPFC after cue-induced reinstatement of cocaine-seeking in *Plcb1*+/- vs wild-type (WT) mice. A Selection of over-represented KEGG pathways (Kyoto Encyclopedia of Genes and Genomes) and (**B**) GO (Gene Ontology) identified by DAVID software among the differentially expressed genes. The number of genes with altered expression included in each category is indicated on the right side of the bar. In red, relevant pathways for the addictive process. **C** Gene network involved in cellular development, cellular growth and proliferation, nervous system development and function (score = 62). The green nodes in the pathway indicate genes with downregulated expression in *Plcb1*+/− identified in RNAseq.

obtained a list of 238 genes that we used for gene network construction. This analysis showed a highly scored network (score = 62, Fig. 3C) that includes 31 DEGs involved in "cellular development, cellular growth and proliferation, nervous system development and function".

DISCUSSION

Here we investigated, for the first time, the contribution of the *PLCB1* gene to cocaine addictive properties using *Plcb1*+/− mice. We found that the heterozygous *Plcb1* genotype resulted in a phenotype of resistance to cue-induced reinstatement of cocaine-seeking behavior. Furthermore, we found relevant transcriptomic differences in *Plcb1*+/- mice compared to WT after cue-induced reinstatement of cocaine-seeking in mPFC, a brain area essential in relapse [35, 36]. This study supports a role for *PLCB1* in cocaine addiction, confirming previous findings in humans [6], and suggests it may be relevant in relapse to cocaine addiction.

The phenotype of *Plcb1*+/− mice was characterized at different levels to evaluate consummatory and general behavior. In general, mutant mice presented normal levels of body weight, food and water intake, locomotor activity, and motor coordination, demonstrating that this animal model is valid to study the effects of the heterozygous deletion of the *Plcb1* gene in other behavioral responses. *Plcb1*+/− showed increased anxiety in the elevated plus maze, reduced short-term memory in the novel object recognition paradigm, without any sign of depressive-like behavior in the anhedonia sucrose preference test. Thus, the single targeting of the *Plcb1* gene has an impact on selective emotional and cognitive responses.

The short-term memory impairment observed in the mutants did not affect the acquisition of operant associative conditioning nor the instrumental learning that drives the goal-directed action, as shown by similar levels of operant cocaine self-administration and extinction learning than WT. In agreement, both genotypes trained with cocaine accomplish cue-induced reinstatement criterion with high enhancement of responding during this test compared to extinction. Thus, different brain circuits are involved in each kind of learning, with perirhinal-hippocampal structures [37] participating in short-term memory in the novel object recognition paradigm and mPFC-dorsal striatum pathway in cue-associated seeking. Also differences in each paradigm such as the acute retrieval or repetitive exposure involved respectively in each task may have a crucial influence in cognition. Hence, cocaine-seeking can be multidimensional, involving different types of associative learning that together lead to an extensive repertoire of conditioned and instrumental responding.

The anxiogenic profile of *Plcb1*+/− is in accordance with the results previously reported in the *Plcb4*−/− mice, which were associated with alterations in the cholinergic activity of the medial septum [38]. However, the selective knock-down of *Plcb1* in the mPFC did not replicate this phenotype in a previous study [39], suggesting that other areas may be involved. The anxiogenic profile of *Plcb1*+/− mice had no effect on cocaine self-administration since the acquisition and extinction of this operant behavior was not modified in the mutants. Besides, mutants showed protection against cue-induced reinstatement of cocaine-seeking, instead of the expected cocaine-seeking promoted by an anxiogenic phenotype [40]. Therefore, the association of an anxiogenic profile with a phenotype resilient to cocaine-seeking in the *Plcb1*+/− mice suggests modifications in specific brain areas involved in cocaine relapse, such as the mPFC. Recently, a phenotype resilient to develop food addiction has been associated with increased strength of pyramidal glutamatergic synaptic transmission in the mPFC related to decreased compulsivity in the face of negative consequences [41] in mice with an anxiogenic profile [42]. Concerning cue-induced cocaine-seeking, the mPFC has a crucial role and the network of glutamatergic projections from the prelimbic mPFC

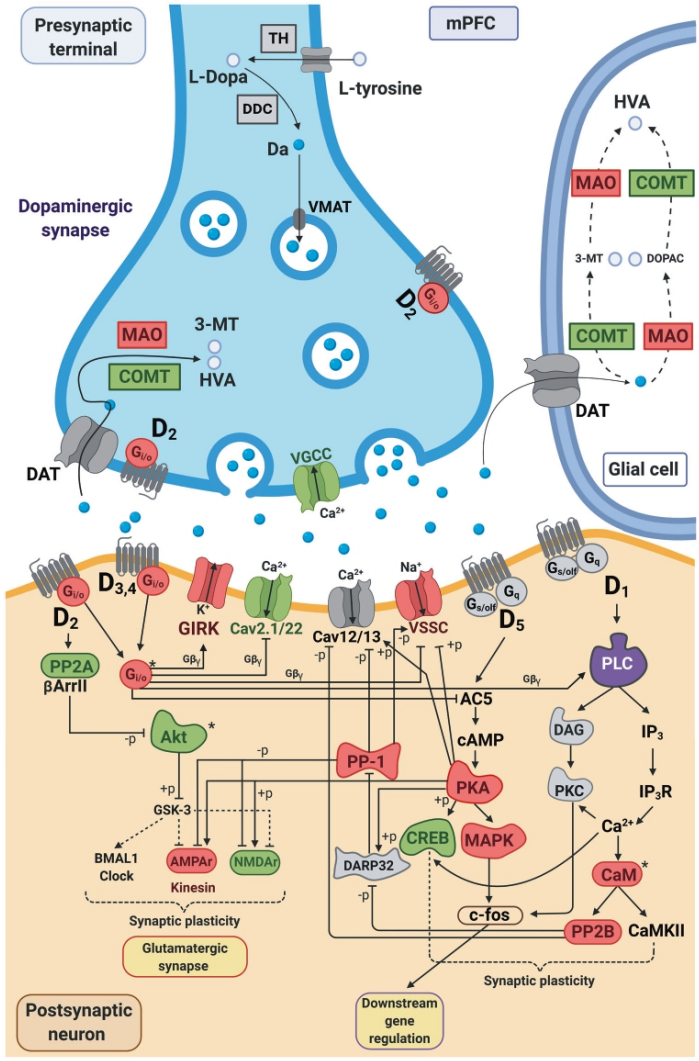

Fig. 4 Alterations in expression in the dopaminergic synapse in mPFC of *Plcb1*+/− mice after cue-induced reinstatement of cocaine-seeking. Adapted from KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways (mmu04728). Enriched pathway in differentially expressed genes in mPFC, 25 out of 131 genes in the pathway were differentially expressed ($P_{raw} = 2e{-}03$; $P_{adj} = 0.02$). In red, upregulated genes and in green, downregulated genes in mPFC. *Protein complexes including upregulated and downregulated genes. Correspondence between genes and proteins can be found in Supplementary Table S6.

to the dorsal striatum participates in this phenotype [43]. In our model, *Plcb1* haploinsufficiency is linked to reduced cue-induced cocaine-seeking possibly associated with modifications in this top-down corticolimbic brain network. Furthermore, these glutamatergic projections from mPFC to dorsal striatum receive mesocortical dopaminergic inputs from the VTA that could be crucially involved in the protective effects of *Plcb1*, since this gene plays an essential role in the dopaminergic signal transmission in the mPFC and many of the genes encoding for proteins in this pathway are differentially expressed in *Plcb1*+/− mice after cue-induced reinstatement (Fig. 4).

Transcriptomic analyses performed after the cue-induced reinstatement of cocaine-seeking behavior revealed some of the molecular underpinnings that underlie the cocaine relapse-related phenotype observed in the mutant mice. Interestingly, differences in gene expression between *Plcb1*+/− and WT were predominantly found in the mPFC, whereas almost no differences were observed in the HPC. This evidence highlights mPFC as a key region to explain these differences in cocaine-seeking reinstatement observed in *Plcb1*+/− mice. Consistently, in DEGs in mPFC, we found enrichment on pathways that are essential for the development of addiction, including the dopaminergic neurotransmission (Figs. 3, 4) [44]. *PLCB1* is part of the dopamine-DARPP-32 signaling pathway (Fig. 4), which plays a key role in cocaine reward [45, 46]. In a previous study, we also found this pathway enriched in DEGs in the frontal cortex and ventral striatum of mice that showed frustrated expected reward, produced by the cue in the absence of expected reward after a high level of effort in a progressive ratio schedule of reinforcement with palatable food [47]. Notably, *Plcb1* was upregulated in the frontal cortex of those frustrated mice with increased responses to obtain the reward (palatable food). These data are in line with our findings in which decreased expression of *Plcb1* (*Plcb1*+/− mice) results in decreased cue-induced responses to obtain cocaine after extinction. All these results support the participation of *Plcb1* in cue-induced cocaine-seeking revealed in the present study and the involvement of mPFC.

Our transcriptomic analyses also found enrichment in genes related to learning and memory processes, such as LTP in the mPFC. The transition from drug use to drug addiction is a maladaptive process that directly affects learning and memory [48, 49]. LTP produces long-lasting activity-dependent synaptic modifications that underlie memory, and drugs of abuse alter this mechanism in several brain regions such as mPFC [50, 51], mesocorticolimbic system [52], VTA [53] and HPC [54, 55], among others. Importantly, genes related to the glutamatergic system such as *Gria2-4* (glutamate ionotropic receptor AMPA2-4 (alpha 2-4)) and *Grik2* (ionotropic glutamate receptor kainate 2 (beta 2)) were found to be upregulated in *Plcb1*+/− mice. Meanwhile, *Grin1, 2c, and 2d* (glutamate ionotropic receptor NMDA1 (zeta 1, epsilon3 and epsilon4)) were downregulated, suggesting an increase in AMPAR/NMDAR ratio in cocaine-experienced and abstinent *Plcb1*+/− mice as compared to WT, one of the key indicators of LTP induction and increased synaptic strength [56]. Furthermore, we found enrichment in other neuroplasticity (synapse organization, neuron projection development, and axon guidance) [14, 57–59] and signaling pathways (MAPK, mTOR, and neurotrophin) [60, 61] related to addiction. The assessment of gene expression in the mPFC of the *Plcb1*+/− mice highlighted alterations in relevant pathways for the addictive process, which could contribute to the results observed in the cue-induced reinstatement.

Nowadays, there are few effective pharmacological treatments available for cocaine use disorders, and frequently, psychosocial interventions in combination with pharmacotherapy are needed [62]. Studies performed in mice have pinpointed multiple potential therapeutic approaches for the reinstatement of cocaine-seeking behavior, targeting the reward circuit [63]. In humans, treatment with bupropion, topiramate, or disulfiram has been widely used. Bupropion, a non-tricyclic antidepressant that inhibits dopamine and norepinephrine reuptake, is effective in reducing craving [64]. Topiramate, a GABA/glutamatergic medication, has also been used to treat cocaine use disorder as it reduces the activity of the mesocorticolimbic dopaminergic system [62]. A completely different strategy is the use of disulfiram which potentially inhibits the oxidoreductase dopamine β-hydroxylase (DβH, encoded by the *DBH* gene), which converts dopamine to norepinephrine [65]. Significantly, a genetic variation in the *SLC6A3* gene (encoding DAT) has been associated with disulfiram treatment for cocaine addiction, with patients with higher DAT levels having better treatment outcomes than those with lower DAT levels [65]. Thus, further studies and new therapeutic targets are needed to obtain effective treatment for cocaine addiction. The results obtained in the present study underscore the relevance of the *Plcb1* gene in the cue-induced reinstatement of cocaine-seeking after extinction. Together with previous findings in humans [6, 7] and mice [8], *PLCB1* merits to be further evaluated as a promising novel therapeutic target for preventing relapse and treating cocaine addiction.

The experimental approach used in our study, the *Plcb1*+/− mouse model, allowed us to reproduce better the molecular context observed in humans in comparison to the use of a complete KO mouse [66], as these animals preserved, at least, half of the expression of *Plcb1*. However, the haploinsufficiency of *Plcb1* during neurodevelopment in these animals could produce alterations that may contribute to the phenotype observed in the present study. Nevertheless, this may also be similar in humans with genetic risk variants that decrease *PLCB1* expression. Therefore, this approach is appropriate to study a specific genetic alteration that confers susceptibility to drug addiction and to delineate the precise contribution of *PLCB1*.

To sum up, we studied, for the first time, the contribution of the *PLCB1* gene to cocaine addictive properties using *Plcb1*+/− mice. Previous studies have revealed an upregulation of *Plcb1* in brain areas related to the reward circuit after cocaine exposure in animals [8] and in human cocaine abusers [6]. These changes, together with our results, suggest that cocaine increases the expression of *Plcb1*, and this mechanism plays an essential role in cocaine addiction, as revealed now by the resistance to cue-induced reinstatement of cocaine-seeking behavior exhibited by *Plcb1*+/− mutant mice. These results highlight the importance of the *Plcb1* gene in the development of cocaine addiction and relapse and pinpoint PLCB1 as a promising therapeutic target for cocaine addiction and perhaps other types of addiction.

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AUTHOR CONTRIBUTIONS

JCD, BC and NFC obtained and maintained the mutant mice (Plcb1+/-); EMG and RM conceived and designed the behavioral studies with input from JCD, BC and NFC; EMG and JCD performed the behavioral phenotype characterization of mutant mice. EMG performed the surgery for i.v. catheterization and the operant conditioning maintained by cocaine. AGR collaborated in extinction, cue-induced reinstatement and the extraction of the samples. EMG and JCD performed statistical analyses and graphs with the supervision of RM, BC and NFC; JCD performed the RNA extractions, RNA sequencing, and the bioinformatic analyses supervised by BC and NFC; EMG and JCD wrote the manuscript and NFC, RM and BC provided a critical review of the manuscript with inputs from all the other authors.

COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Noèlia Fernàndez-Castillo or Bru Cormand.

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