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EXPLORING NEW PHARMACOLOGICAL STRATEGIES TO TREAT BINGE-LIKE EATING BEHAVIOR

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SUMMARY

Binge eating disorder (BED) is the most common eating disorder. It is characterized by recurrent episodes of binge eating, during which individuals consume, in a discrete period of time, amounts of food larger than most people would eat under similar circumstances, while experiencing loss of control towards their eating behavior. BED has a strong negative impact on the functionality and quality of life, and it is associated with negative health consequences, including obesity and other psychiatric disorders. Psychological therapy is generally recommended for the treatment of BED, but unfortunately not all of the patients positively respond to this approach. Thus, pharmacological approaches are strongly required for the management of this pathology. To date, only Lisdexamfetamine dimesylate (LDX) has been approved for the treatment of BED by the Food and Drug Administration (FDA) in 2015, even though its clinical efficacy is limited by the adverse effects associated to this psychostimulant, in particular the increase in heart rate and blood pressure, which might lead to cardiovascular adverse events. The research for innovative pharmacological strategies is necessary, and the development of animal models of binge eating is a useful approach to investigate the neurobiological processes underlying binge eating behavior and to test the efficacy of innovative compounds to block binge eating episodes. Based on this premises, in this experimental thesis I tested the effects of two innovative compounds which might represent useful pharmacological agents for the treatment of binge eating behavior, and I also investigated potential neurobiological alterations underlying compulsive-like eating behavior, using an animal model of binge eating developed by Cifani et al. in 2009. This animal model involves the use of female rats, which develop a compulsive-like eating behavior towards highly palatable food (HPF), after being exposed to cycles of

caloric restriction/refeeding plus a frustration stress procedure. This animal model is characterized by a high degree of face, construct and predictive validity. The first target analyzed in this thesis is the Sigma 1 receptor (σ1R), a chaperone-like protein which has been demonstrated to promote binge eating behavior. In particular, I tested the effect of a highly selective σ1R antagonist in the binge eating model of Cifani et al., observing that this compound was able to block the binge eating episode in female rats, without affecting anxiety-like and depressive-like behaviors. Thus, the results of this study evidenced that antagonism of the σ1R is a pharmacological strategy to selectively target and block the neuronal mechanisms that lead to the binge eating episode. The second target of my experimental thesis is orexin-A (OX-A) and the orexin-1 receptor (OX1R), importantly implicated in driving non-homeostatic consumption of HPF. Specifically, I tested the effect of a selective OX1R antagonist (SO1RA) developed by Idorsia Pharmaceuticals Ltd, named ACT-539313, on binge eating behavior. This compound blocked the binge eating episode in female rats under both acute and chronic administration regimens, without sign of tolerance. I also investigated by immunohistochemistry whether binge eating rats might display alterations in Orexin-A (OX-A) and Delta FosB protein expression (marker of long-term neuronal adaptation and plasticity) in multiple brain regions implicated in binge eating behavior. Rats chronically exposed to the binge eating protocol displayed a down-regulation of OX-A protein expression in different extra-hypothalamic sites, such as the central amygdala (CeA), dorsal raphe nucleus (DRN) and paraventricular nucleus of the thalamus (PVT), which could represent a protective mechanism against overconsumption of palatable food. In contrast, no changes in Delta FosB protein expression were found in all brain regions analyzed in binge eating rats. I also investigated whether chronic administration of ACT-539313 might produce changes

in the expression of OX-A and delta FosB. Chronic treatment with the SO1RA led to an increase in the number of OX-A positive neurons in the hypothalamus, reflecting a compensatory increase in OX-A expression in response to the SO1RA, and also to an increase in the number of hypothalamic delta-FosB positive cells, suggesting activation of populations of neurons in the hypothalamus. Collectively, these results support the use of SO1RAs in the treatment of binge eating, with efficacy observed even under chronic administration regimen. Furthermore, changes in OX-A protein expression at extra-hypothalamic brain regions appear to represent a marker of binge eating behavior. In conclusion, the data obtained in my experimental thesis provide novel insights about the potential role of the σ1R and OX1R in driving binge eating behavior, and support the clinical evaluation of antagonists of these receptors in humans affected by BED.

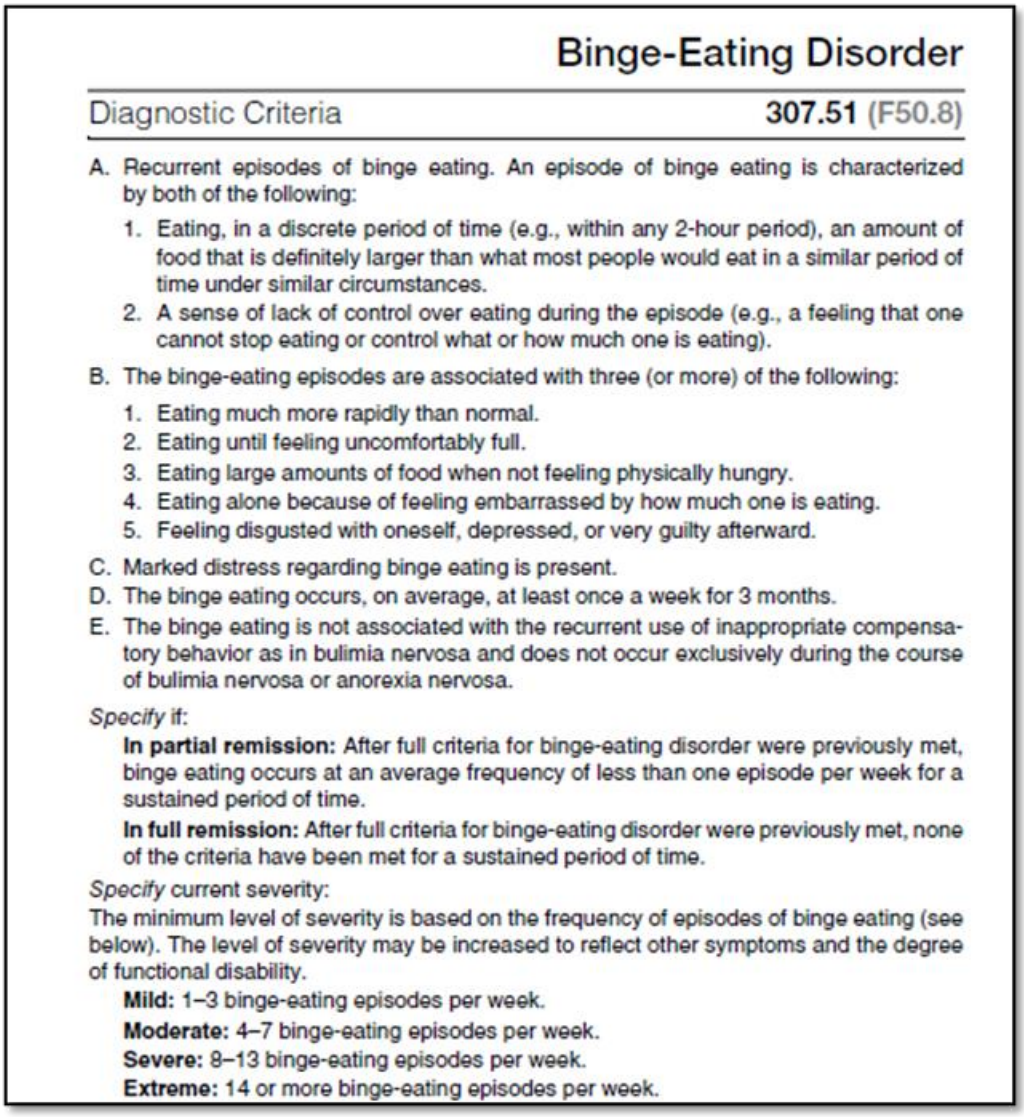
1. GENERAL INTRODUCTION

1.1. Binge Eating Disorder (BED)

Binge Eating Disorder (BED) is the most common eating disorder and an important public health problem worldwide. Originally, BED was introduced in the appendix of the Diagnostic and Statistical Manual of Mental Disorders, 4th Edition, Text Revision (DSM-IV-TR), as a subcategory of Eating Disorders Not Otherwise Specified (EDNOS). Subsequently, it was recognized as a discrete eating disorder in the Diagnostic and Statistical Manual of Mental Disorders, Edition 5 (DSM-V), in 2013 (American Psychiatric Association & Association, 2013). The main psychopathological feature characterizing BED is the presence of regular binge eating episodes, during which individuals consume, in a discrete period of time (about 2 hours), amounts of food larger than most people would eat under similar circumstances, while experiencing loss of control towards their eating behavior (American Psychiatric Association & Association, 2013). BED and Bulimia Nervosa (BN) are both characterized by the presence of regular binge eating episodes, but differently from BN, BED patients do not engage in one or more inappropriate compensatory behaviors, such as self-induced vomiting, excessive exercise, use of diuretics or laxatives (American Psychiatric Association & Association, 2013; Guerdjikova, Mori, et al., 2017). According to the DSM-5, to fulfil the diagnostic criteria for BED, the binge eating episodes have to be associated with feelings of guilt and marked distress after over-eating and have to occur at least once a week for at least three months (American Psychiatric Association & Association, 2013; Giel et al., 2022; Guerdjikova, Mori, et al., 2017). The DSM-5 describes these episodes to be accompanied by three of the following five clinical features: eating much more rapidly than normal, eating until feeling uncomfortably full, eating alone because of the shame

and embarrassment for the amount ingested, eating without being physically hungry, and perception of negative feelings after over-eating (American Psychiatric Association & Association, 2013). The DSM-5 also defines the severity rating of BED ranging from Mild (1–3 episodes/week) to extreme (14 or more episodes/week) (American Psychiatric Association & Association, 2013; Heal & Smith, 2022). The diagnostic criteria and severity ratings of DSM-5 BED are illustrated in Table 1.

Table 1. Diagnostic Criteria for BED in the DSM-5. (American Psychiatric Association & Association, 2013).



Binge-Eating Disorder

Diagnostic Criteria **307.51** (F50.8)

A. Recurrent episodes of binge eating. An episode of binge eating is characterized by both of the following:
   1. Eating, in a discrete period of time (e.g., within any 2-hour period), an amount of food that is definitely larger than what most people would eat in a similar period of time under similar circumstances.
   2. A sense of lack of control over eating during the episode (e.g., a feeling that one cannot stop eating or control what or how much one is eating).

B. The binge-eating episodes are associated with three (or more) of the following:
   1. Eating much more rapidly than normal.
   2. Eating until feeling uncomfortably full.
   3. Eating large amounts of food when not feeling physically hungry.
   4. Eating alone because of feeling embarrassed by how much one is eating.
   5. Feeling disgusted with oneself, depressed, or very guilty afterward.

C. Marked distress regarding binge eating is present.

D. The binge eating occurs, on average, at least once a week for 3 months.

E. The binge eating is not associated with the recurrent use of inappropriate compensatory behavior as in bulimia nervosa and does not occur exclusively during the course of bulimia nervosa or anorexia nervosa.

Specify if:

In partial remission: After full criteria for binge-eating disorder were previously met, binge eating occurs at an average frequency of less than one episode per week for a sustained period of time.

In full remission: After full criteria for binge-eating disorder were previously met, none of the criteria have been met for a sustained period of time.

Specify current severity:

The minimum level of severity is based on the frequency of episodes of binge eating (see below). The level of severity may be increased to reflect other symptoms and the degree of functional disability.

Mild: 1–3 binge-eating episodes per week.

Moderate: 4–7 binge-eating episodes per week.

Severe: 8–13 binge-eating episodes per week.

Extreme: 14 or more binge-eating episodes per week.

BED continues to be underrecognized and undertreated, but it is a very important health issue and highly frequent in the general population (Giel et al., 2022; Guerdjikova et al., 2019; Keski-Rahkonen, 2021). In 2013, data from the World Health Organization Mental Survey Study, performed across 14 countries, revealed a lifetime prevalence rate of BED of 1.4 %, even consistently higher than for BN (0.8 %), and BED was found more frequent in females than males (Kessler et al., 2013). Recently, BED was estimated to affect 1.5% of women and 0.3% of men worldwide, with a

lifetime diagnosis of DSM-5 BED reported to be 0.6-1.8% in women and 0.3-0.7% in men (Keski-Rahkonen, 2021). With the onset of the novel coronavirus disease (COVID-19), the burden of eating disorders was further aggravated, mostly due to the stress, anxiety, depression, and changes in eating habits linked to the lockdown restrictions (Mumtaz et al., 2022). In particular, the diagnostic incidence of eating disorders was found 15.3% higher in 2020 compared to the previous years, concomitant with the COVID-19 pandemic (Taquet et al., 2021). BED is highly prevalent in children and adolescents, with a peak frequency at the age of 16-17 years, and treatment of BED in youth is of vital importance, since adolescents with BED have increased risk of obesity, substance use, suicidality, and other mental disorders (Bohon, 2019; Marzilli et al., 2018). BED is generally associated to multiple comorbid conditions, including both psychiatric and non-psychiatric diseases, which are commonly the primary focus of the treatment, and thus BED can often be unrecognized and undertreated (Citrome, 2017; Mitchell, 2016). Considering the intrinsic nature of binge eating behavior, BED is strongly linked with elevated body mass index (BMI), obesity and even extreme obesity. For example, a large US nationally representative survey demonstrated that lifetime diagnosis of BED was significantly associated with current severe obesity (Hudson et al., 2007). An earlier onset of being overweight and history of obesity have also been observed in BED patients (Jacobi et al., 2004; McCuen-Wurst et al., 2018). In addition, BED is commonly correlated with obesity-associated clinical complications, such as hypertension, metabolic syndrome, and type 2 diabetes (Guerdjikova et al., 2019; Harris et al., 2021; McCuen-Wurst et al., 2018). In the general population BED appears to be also accompanied by a range of lower and upper gastrointestinal symptoms, including acid regurgitation, heartburn, dysphagia, bloating, upper abdominal pain, diarrhea, urgency, constipation and feeling

of anal blockage (Cremonini et al., 2009). BED often co-occurs with other mental health issues. In a nationally representative sample of adults in the US, 93.8 % of individuals with BED met criteria for at least one additional psychiatric disorder (Udo & Grilo, 2019). Furthermore, BED had the greatest number of associations with other psychiatric disorders, in comparison with Anorexia Nervosa and BN (Udo & Grilo, 2019). Among patients with BED, the most common psychiatric comorbid conditions reported are mood disorders, anxiety, post-traumatic stress disorder, and substance use disorders (Citrome, 2017; Guerdjikova et al., 2019; Lydecker & Grilo, 2021; Udo & Grilo, 2019). The presence of psychiatric comorbidity is particularly relevant, since it is correlated with a more severe BED psychopathology, higher binge eating frequency, and difficulty in remission (Lydecker & Grilo, 2021). Poor impulse control, high degree of general impulsivity and impulsivity-related psychopathology have been additionally observed in BED patients, supporting that impairment of impulse control might represent an important target in the management of BED (Boswell & Grilo, 2021; Carr et al., 2021).

1.2. Neurobiology of BED: evidence from human studies

Despite its high prevalence, the etiology, the pathophysiology, and the neurobiological features underlying BED are understudied and not completely understood. BED is a complex and multifactorial disease, in which several factors might participate and contribute to the etiopathogenesis, including genetics and neurobiological processes, as well as environmental and sociodemographic conditions. Even though being frequently associated with obesity, BED has a distinct phenotype and might be conceptualized as an impulsive/compulsive disorder (Boswell et al., 2021). A high

sensitivity to food reward coupled with elevated impulsivity and compulsivity are observed in BED patients, potentially reflecting an altered functioning of the mesocorticolimbic dopamine system (Balodis et al., 2015; Boswell et al., 2021; Kessler et al., 2016). Using functional near-infrared spectroscopy, it has been recently reported that individuals with BED, under response inhibition tasks, show an attenuated recruitment of the prefrontal cortex (PFC) (Rosch et al., 2020; Veit et al., 2021), brain region involved in reward- and value-based decision making, self-regulation and control of food intake (DelParigi et al., 2007; Hiser & Koenigs, 2018). The weaker activation of the PFC was correlated with higher traits of impulsivity (Veit et al., 2021). Interestingly, after 3 months of impulsivity-focused cognitive behavioral therapy (CBT), BED patients reported an improvement in controlling impulsive behaviors, concomitant with a more pronounced activation of the PFC (Veit et al., 2021). The response-inhibition tasks require the recruitment of prefrontal control network, but this neural process seems to be impaired in BED subjects, when compared to overweight individuals without BED, and this is correlated with high attentional impulsiveness scores (Hege et al., 2015). Functional disturbances in brain regions implicated in self-control were also demonstrated in a study of Balodis et al., which reported diminished activity of the ventromedial PFC, inferior frontal gyrus and insula in BED patients, compared to obese and lean individuals without BED, during a Stroop color-word interference task (a test that measures the ability to inhibit pre-potent response tendencies) (Balodis, Molina, et al., 2013). Similarly, a reduced cortico-striatal processing was observed in BED individuals across both the anticipatory and outcome phases of a monetary reward/loss task (Balodis, Kober, et al., 2013). Altogether, these studies highlight a reduced recruitment of cortico-striatal networks in BED, resulting in impairment of neuronal circuitries involved in reward

processes, impulsivity, and self-regulation. This generalized pattern of diminished fronto-striatal activity in BED patients supports a hyposensitivity towards non-food rewards, that, combined with a hypersensitivity to food rewards might precipitate over-eating and binge eating behavior (Boswell et al., 2021). Indeed, food cue-induced craving and craving for highly palatable food (HPF) are higher in obese individuals with BED compared to those without (Reents & Pedersen, 2021). An increase in reinforcement sensitivity in BED has been additionally found in a neuroimaging study of Schienle et al., in which female BED patients revealed high self-reported reward responsiveness, that was positively associated with a greater activation of the orbitofrontal cortex (OFC), compared to females with BN and healthy control subjects, in response to food cues (Schienle et al., 2009). The OFC, a secondary gustatory cortex (Rolls et al., 1990), integrates afferent and efferent projections including prelimbic, limbic and sensory regions, with an important role in decision making related to feeding behavior, and in the hedonic and rewarding aspect of palatable food consumption (Seabrook & Borgland, 2020). In line with these findings, when exposed to high-calorie taste cues, individuals suffering of compulsive over-eating display activation of several brain regions of the reward system, including the medial OFC, ventral tegmental area (VTA), insula, caudate, putamen, nucleus accumbens (NAc), and precuneus (Filbey et al., 2012). An increased neural response is also observed in regions underlying emotional processes, such as the amygdala and hippocampus (Filbey et al., 2012). Interestingly, the hyper-responsivity to high-calorie taste cues positively correlates with the intensity of binge eating symptoms (Filbey et al., 2012). Consistently, a study of Wolz et al. directly examined neuronal activity in individuals with binge eating, using electroencephalography and chocolate cues (odor and images), registering stronger event-related potentials to chocolate in these subjects,

compared to healthy controls (Wolz et al., 2017). Summarizing, these studies evidenced a hyper-responsivity of specific brain regions (principally of the reward system) in individuals suffering of BED in response to palatable food and food-related stimuli, which might explain their tendency to compulsively over-eat. However, contrasting results have been reported in literature. For example, a recent functional MRI (fMRI) study revealed a diminished pattern of neural responding for food stimuli in individuals who binge eat (Donnelly et al., 2022). Thus, the precise neurobiology of BED remains uncertain and ongoing, and additional research is necessary for a better understanding of the neural mechanisms implicated in this psychiatric disorder. Generally, most of the research point to an altered functioning of different brain networks, in particular the hypothalamus, implicated in the regulation of metabolic signals and motivational circuitries, and the cortico-striatal circuitries, which regulate motivated behavior for rewarding stimuli (including food) and are concerned with inhibitory control (Boswell et al., 2021; Giel et al., 2022; Kessler et al., 2016). The principal brain structures implicated in BED are illustrated in Figure 1.

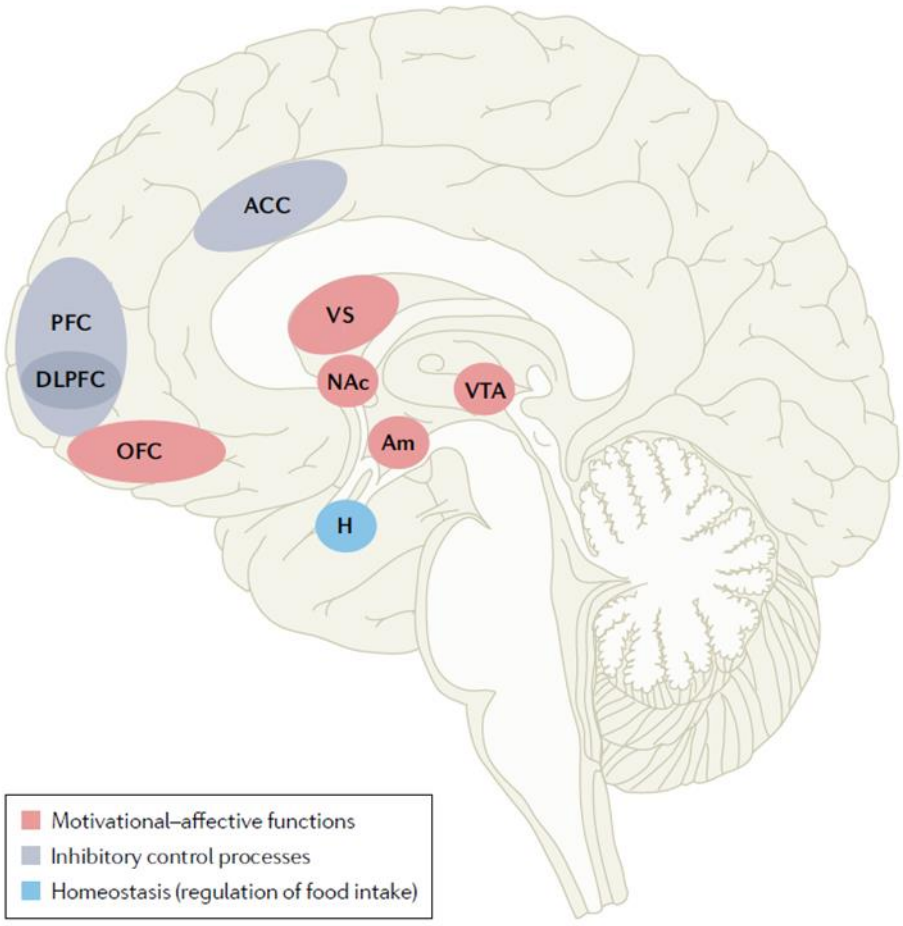


Figure 1. The most representative brain regions implicated in BED (Giel et al., 2022). Generally, three principal neuronal circuitries appear to participate in the pathophysiology of BED: the hypothalamus, implicated in the regulation of energy homeostasis and food intake regulation; the reward system, which includes the ventral striatum, NAc, VTA, the amygdala and the OFC, and it is implicated in motivational processes; the cortical structures such as the PFC, the dorsolateral PFC and the anterior cingulate cortex, implicated in impulse control regulation. ACC, anterior cingulate cortex; Am, amygdala; DLPFC, dorsolateral prefrontal cortex; H, hypothalamus; NAc, nucleus accumbens OFC, orbitofrontal cortex; PFC, prefrontal cortex; VS, ventral striatum; VTA, ventral tegmental area.

1.3. The principal neurotransmitter systems implicated in BED

1.3.1. Dopamine

The neurotransmitter dopamine has been always considered particularly relevant in the neurobiology of binge eating behavior, considering the wide distribution of dopamine receptors, and its role in food craving, executive function, decision making and impulse control (Avena & Bocarsly, 2012; Bello & Hajnal, 2010; Naef et al., 2015; Yu et al., 2022). In the brain, four main dopaminergic pathways have been identified: 1) the mesolimbic, involving projections from the VTA to the ventral striatum; 2) the mesocortical, consisting in projections from the VTA to the PFC; 3) the nigrostriatal, originating in the substantia nigra and projecting to the dorsal striatum; 4) the tuberoinfundibular, projecting from hypothalamic nuclei to the pituitary (Bjorklund & Dunnett, 2007; Klein et al., 2019). These pathways are represented in Figure 2.

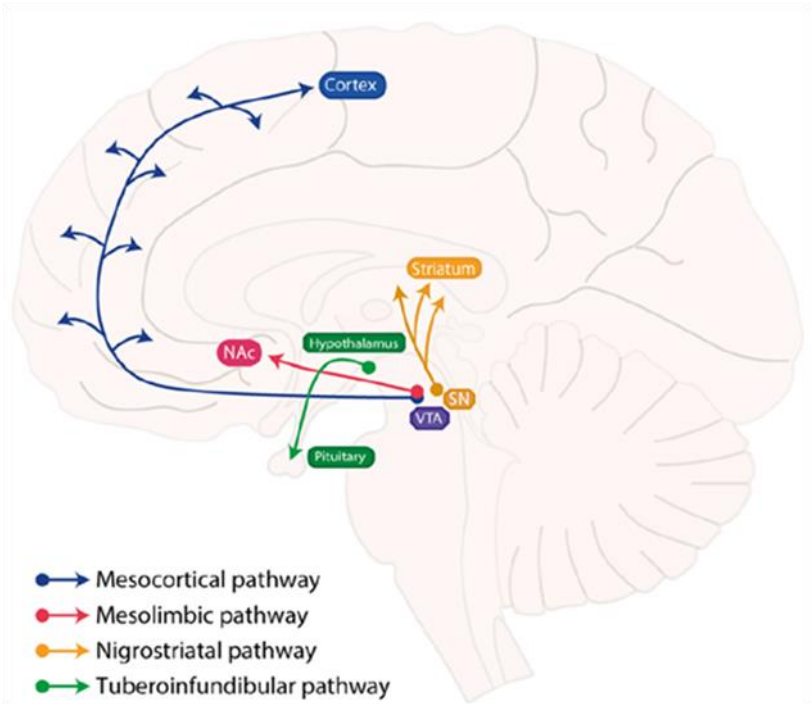


Figure 2. The four main dopaminergic pathways in the central nervous system. Adapted from (Klein et al., 2019). NAc, nucleus accumbens; SN, substantia nigra; VTA, ventral tegmental area.

Dopamine is crucially implicated in food consumption (Palmiter, 2007), and it is required for motivation and reward. In particular, dopaminergic projections from the VTA to the NAc and PFC are implicated in what is generally referred as the brain "wanting" system (Berridge, 2009; Volkow et al., 2017; Wise, 2006). Animal models of binge eating have shown that an intermittent access to a palatable sugar solution promotes binge-like eating in rats, causing the repeated release of dopamine in the NAc (Avena & Bocarsly, 2012; Avena et al., 2008). The excessive sugar intake is accompanied by dysregulation of the mesolimbic dopamine system, observing an increased binding to the dopamine receptor D1 (DRD1) in the NAc, increased binding to the dopamine transporter (DAT) in the midbrain, and a decrease in DRD2-binding in the dorsal striatum (Colantuoni et al., 2001). Similar findings were replicated in other studies, supporting the lower binding to the DRD2 and the upregulation of the DAT in rats under daily limited access to sucrose (Bello et al., 2002; Bello et al., 2003). A hypo-functionality of the brain reward system has been additionally reported in rats that volitionally over-eat a palatable "cafeteria" diet, which display a reduced striatal expression of the DRD2 (Johnson & Kenny, 2010). This suggests that palatable food consumption might promote plasticity of the reward system, and deficits in striatal DRD2 expression appears to increase the vulnerability to compulsively over-eat (Johnson & Kenny, 2010). It is interesting to note that the molecular and neurochemical adaptations observed in response to excessive consumption of palatable food are similar to those observed in drug addiction. This supports the idea that binge eating might be considered a form of addictive-like behavior (Kenny, 2011; Volkow et al., 2017) and the hypothesis of "food addiction", firstly introduced in the scientific literature in 1956 (Lindgren et al., 2018; Randolph, 1956). Accordingly, in humans, a positron emission tomography (PET) study revealed that obese subjects with BED

have greater extracellular dopamine levels in the caudate nucleus in response to food stimuli, in comparison with obese individuals without BED (Wang et al., 2011). Dopamine extracellular levels also positively correlated with higher binge eating scores (Wang et al., 2011). Moreover, polymorphic variants of the genes encoding the DRD2 and the DRD4 have been frequently associated with an increased risk of developing aberrant feeding patterns and BED (Botticelli et al., 2020; Ceccarini et al., 2022; Davis et al., 2012).

1.3.2. Serotonin

A potential role for serotonin in the neurobiology of binge eating has been proposed in literature. Firstly, serotonin signaling affects both the homeostatic and hedonic aspects of eating behavior (van Galen et al., 2021), and obesity appears to be linked with a decreased serotoninergic activity (van Galen et al., 2018). Secondarily, drugs that stimulate the central serotonin transmission are potent appetite suppressants (Nigro et al., 2013; Shinozaki et al., 2008; Uphouse et al., 2006). In human, imaging studies suggest that patients with BED are characterized by a regionally selective up- and down-regulation of the serotonin transporter (SERT). Specifically, it is reported an increased SERT-binding in parieto-occipital cortical regions, together with a decreased binding in regions of the reward system, such as the NAc, inferior temporal gyrus, lateral OFC and in the midbrain (Kuikka et al., 2001; Majuri et al., 2017). In mice, administration of drugs that enhance brain serotonin content (such as fluoxetine or d-fenfluramine), or that directly stimulate the serotonin receptors (lorcaserin), was reported to suppress binge-like eating, in a mechanism specifically mediated by the 5-hydroxytryptamine$_{2c}$ (5-HT$_{2c}$) receptors located on dopaminergic neurons of the VTA

(Xu et al., 2017). Moreover, in rats, pharmacological activation of 5-HT_{2c} receptors demonstrated efficacy in suppressing binge-like eating for high fat food and operant responding for palatable food pellets (Price, Anastasio, et al., 2018; Price, Brehm, et al., 2018).

1.3.3. Opioids

Endogenous opioid peptides comprise endorphins, enkephalins, dynorphins and endomorphins, which act through three types of receptors: μ-, δ- and κ-opioid receptors (MOR, DOR, and KOR), all members of the G protein-coupled receptor (GPCR) superfamily (Valbrun & Zvonarev, 2020). The opioid system is strongly implicated in the rewarding aspect of palatable food intake, and the opioid neurotransmission is an important mediator of hedonic overeating (Giuliano & Cottone, 2015; Nathan & Bullmore, 2009). Indeed, binge-like eating for a sucrose solution in rats was found to increase μ-opioid receptor-binding in several brain structures, including the NAc (Colantuoni et al., 2001), and the deletion of μ-opioid receptors in mice results in lower levels of food-driven operant behavior under progressive ratio schedules (Papaleo et al., 2007). The injection of μ-opioid receptor agonists has been demonstrated to drive palatable food intake, and to increase the consumption of a preferred food when also the less preferred one was available at the same time (Woolley et al., 2006). On the other hand, μ-opioid receptor antagonists were reported to decrease binge eating in both animal models and humans (Blasio et al., 2014; Cottone et al., 2008; Giuliano et al., 2012; Ziauddeen et al., 2013). The effect of opioid receptors antagonists on food intake was found dependent on the site of administration. Indeed, naltrexone, when injected in the NAc, has a general

suppressive effect on food consumption, while when injected in the medial PFC, it selectively decreases consumption and motivation for palatable food in bingeing rats (Blasio et al., 2014). Another selective μ-opioid receptor antagonist, named GSK1521498, proved to decrease food seeking and binge-like eating in rats, affecting both the hedonic and the incentive motivational aspect of palatable food consumption (Giuliano et al., 2012). The same compound was tested in human studies and was able to decrease the hedonic preference for sugar and fat, the calorie intake from palatable food, and neural response to food-related images in obese subjects with BED (Cambridge et al., 2013; Ziauddeen et al., 2013).

1.3.4. Endocannabinoids

The endogenous cannabinoid system critically modulates feeding behavior by increasing motivation for food seeking and intake, influencing the mesolimbic reward pathway, and modulating the activity of orexigenic and anorexigenic hormones at a hypothalamic level (D'Addario et al., 2014; Di Marzo et al., 2009; Vemuri et al., 2008). The endocannabinoids are derivatives of the arachidonic acid, and they can be conjugated with ethanolamine (forming the fatty acid amides) or with glycerol (forming the monoacylglycerols). N-arachidonoylethanolamine (anandamide, AEA) and 2-arachidonoylglycerol (2-AG) are the most active members of these classes (Devane et al., 1992; Mechoulam et al., 1995), and they activate two well-characterized GPCR subtypes: the cannabinoid receptor type 1 (CB1) and type 2 (CB2) (Herkenham et al., 1991; Mackie, 2008). It was soon reported that the stimulation of CB1 receptors produces orexigenic effects, while pharmacological antagonism was associated to a decrease in appetite (Carai et al., 2006; Di Marzo et al., 2009; Salamone

et al., 2007). This led to the development and approval of the CB1 receptor antagonist Rimonabant for the treatment of obesity, by Sanofi-Aventis. However, despite Rimonabant' efficacy in the obesity management, the use of this compound was associated to severe psychiatric side effects (anxiety, depression and suicidal ideations), and it was soon removed from the market (Moreira & Crippa, 2009; Samat et al., 2008). Neuroadaptations of the endocannabinoid system have been reported in animal models of binge eating (Bello et al., 2012; de Sa Nogueira et al., 2021; Satta et al., 2018). For example, a recent study evidenced an up-regulation of CB1 receptor gene expression in the NAc, increased AEA levels in the PFC, and decreased 2-AG levels in the hippocampus of rats bingeing for sucrose (de Sa Nogueira et al., 2021). Moreover, in a rat model of binge eating with an intermittent and limited access to margarine, decreased levels of AEA in the caudate putamen, amygdala and hippocampus, and increased levels of 2-AG in the hippocampus were detected in bingeing rats (Satta et al., 2018). Intriguingly, in these animal models, the CB1 receptor antagonist Rimonabant demonstrated the ability to suppress binge-like eating and conditioned place preference for palatable food (de Sa Nogueira et al., 2021; Scherma et al., 2013). In addition to the classical endocannabinoids, endocannabinoid-like lipids, such us the oleoylethanolamide (OEA), acting as ligand for the peroxisome proliferator-activated receptor-α (PPAR-α), are implicated in eating behavior. This lipid was reported to inhibit food intake (Provensi et al., 2014; Romano et al., 2017; Romano et al., 2014), and to block the episode of binge eating in female rats (Romano et al., 2020).

1.4. Pharmacological approaches for BED

The international guidelines recommend psychological therapy as the primary approach to treat individuals with BED (Guerdjikova et al., 2019; Hilbert et al., 2017; National Guideline, 2017). Three main therapies have proved efficacy in the treatment of BED from randomized controlled trials: CBT, interpersonal psychotherapy (IPT) and dialectical behavioral therapy (DBT) (Fairburn, 2008; Wilfley et al., 2002; Wisniewski, 2010). However, not all patients respond to psychological approaches, and thus, various drug agents have been evaluated as potential pharmacological tools for the management of BED (Appolinario et al., 2019; Heal & Smith, 2022; McElroy, 2017). The pharmacological properties and characteristic of the "ideal" drug to treat BED are described in a recent review (Heal & Smith, 2022), and summarized in Table 2.

Table 2. Characteristics of the "ideal" drug for the treatment of BED. Adapted from (Heal & Smith, 2022).

Characteristics of the ideal drug for BED:
Prevent the incidence of uncontrolled BE episodes.
Reduce impulsive, compulsive and perseverative symptoms of BED.
Increase cognitive restraint over food consumption.
Restore healthy eating patterns.
Reduce bodyweight to help overweight/obese subjects achieve a healthy BMI.
The weight-loss effect should not be so powerful that it causes large decreases in BMI in normal weight subjects with BED.
Reduce food intake by increasing satiety not by suppressing appetite thereby disrupting normal meal patterns.
It should be safe when used long term.
The 'ideal' drug should not:
Produce pharmacological tolerance that would result in dose-escalation.
Cause psychological or physical dependence.
Be subject to human abuse.
Be a controlled drug.

In this section it is reported an overview of drugs that are currently available for the treatment of BED, and of drug agents that demonstrated promising results in clinical

and preclinical studies, representing potential future pharmacological tools for this psychiatric disorder.

1.4.1. Lisdexamfetamine dimesylate (LDX)

Currently, the only approved drug for the treatment of BED is LDX (Vyvanse®, Takeda), approved by the United States Food and Drug Administration (FDA) in 2015 (Food & Administration, 2015). Pharmacologically, LDX is an inactive pro-drug of d-amphetamine, covalently linked with the naturally occurring amino-acid l-lysine. LDX is metabolized into the active form (d-amphetamine) through an enzymatic hydrolysis process mostly associated with the red blood cells (Hutson et al., 2014; Pennick, 2010; Sharman & Pennick, 2014). In vitro studies demonstrated that d-amphetamine is a moderately potent inhibitor of DAT, noradrenaline transporter (NET), vesicular monoamine transporter 2 (VMAT2), and it has weaker affinity for SERT. Additionally, d-amphetamine is a weak inhibitor of monoamine oxidases (MAO). The general effect observed is an enhanced catecholamine availability in the extracellular space, through the reversal of catecholamine transport out of the neuronal terminal (Heal et al., 2013; Hutson et al., 2014). In a preclinical model in which binge eating is elicited in female rats by giving unpredictable, intermittent access to ground, milk chocolate over four weeks, LDX proved to dose-dependently decrease excessive chocolate consumption, without affecting the intake of standard chow (Vickers et al., 2015). In the same model, LDX administration normalized the compulsive and perseverative behaviors, and the cognitive impulsivity observed in bingeing rats (Heal et al., 2016; Vickers et al., 2017). More recently, Presby et al. reported that intraperitoneal (i.p.) injection of LDX led to a suppression of both chocolate and chow

intake in rats exposed to the more palatable food (Presby et al., 2020). Additionally, LDX reduced food-reinforced lever pressing in a task in which rats had a choice between working for chocolate pellets or consuming an available but less preferred normal chow (Presby et al., 2020). In clinical trials, LDX (at doses of 50 and 70 mg/kg) demonstrated the ability to decrease the number of binge eating days/week relative to placebo in adults with moderate to severe BED (McElroy et al., 2016; McElroy et al., 2015), and to reduce the risk of relapse in patients continuing LDX treatment for longer periods (Hudson et al., 2017). Finally, eight-weeks treatment with LDX resulted not only in a reduction of binge eating frequency, but also in a concomitant decrease of self-reported impulsivity (Griffiths et al., 2021). However, despite the promising results obtained from both preclinical and clinical studies, LDX, as a central nervous system stimulant, has a risk for abuse and dependence. Moreover, its administration is commonly associated with adverse effects, such as dry mouth, decreased appetite, insomnia, feeling jittery, constipation, anxiety, and enhanced heart rate. For this reason, LDX should not be administered in patients suffering of cardiomyopathies (Coghill et al., 2014; McElroy et al., 2016; Ward & Citrome, 2018).

1.4.2. Promising future pharmacological treatments for BED: results from clinical trials

<u>Dasotraline:</u>

Similar to LDX, dasotraline has a catecholaminergic mechanism. It is a potent inhibitor of the human DAT (dopamine uptake IC50 3 nM), NET (norepinephrine uptake IC50 4 nM), and a weaker inhibitor of SERT (serotonin uptake IC50 15 nM) (Koblan et al., 2016). The pharmacokinetic profile of dasotraline highlights a slow absorption and a

long elimination half-life (T1/2 47-77 h), resulting in stable plasma levels over 24 h, and once-daily administration (Hopkins et al., 2016; Koblan et al., 2015). The efficacy and safety of dasotraline have been evaluated in two placebo-controlled double-blind clinical trials in adults with BED (Grilo, McElroy, et al., 2021; McElroy et al., 2020). In the first clinical trial, once-daily treatment with flexible doses of dasotraline (4, 6 and 8 mg) significantly decreased the number of binge eating days per week, compared to the placebo. Dasotraline administration also produced a general improvement in behaviors representing the core psychopathology of BED (McElroy et al., 2020). In the second clinical trial, two fixed doses (4 and 6 mg) of dasotraline were evaluated in adults with BED. At week twelve of treatment, dasotraline at 6 mg/day, but not at 4 mg/day, produced a significant reduction in the number of binge eating days per week (Grilo, McElroy, et al., 2021). However, an improvement of the overall illness severity was obtained with both doses of dasotraline (Grilo, McElroy, et al., 2021). The drug was relatively safe and generally well-tolerated in BED patients in both two studies, with the most common adverse effects being insomnia, dry mouth, decreased appetite, anxiety, nausea, decreased weight and headache. Changes in blood pressure and pulse were minimal (Grilo, McElroy, et al., 2021; McElroy et al., 2020). However, despite these promising results and the pre-registration of dasotraline for adults with BED in the USA, the company Sunovion announced to not continue the development of dasotraline for this psychiatric disorder, because additional clinical studies would have been necessary for the regulatory approval. Thus, the company decided to not invest in further development of the drug.

Glucagon-like peptide 1 (GLP-1) receptor agonists:

GLP-1 is an incretin hormone secreted by the endocrine L cells of the distal intestinal mucosa, primarily in the ileum and distal colon. It promotes a decrease in blood glucose, by increasing the levels of insulin and decreasing glucagon secretion (Drucker, 2018; Holst, 2007; Ladenheim, 2015). GLP-1 also regulates food intake by delaying gastric emptying and through inhibition of appetite centers in the brain (Holst, 2007). Liraglutide, developed by Novo Nordisk, is a long acting GLP-1 receptor agonist that has been approved by the European Medicines Agency (EMA) and FDA for the treatment of diabetes (Knudsen & Lau, 2019). The FDA also approved this drug for chronic weight management in adults with a BMI of 30 kg/m^2 or higher or a BMI of 27 kg/m^2 or higher who have at least one weight-related condition (Ladenheim, 2015). Considering the beneficial effects of liraglutide in obesity and diabetes, this drug was also investigated in BED. In a study performed in obese non-diabetic binge eaters, participants who received liraglutide for twelve weeks had a significant improvement in binge eating (assessed by the validate questionnaire Binge Eating Scale), accompanied by a reduction in body weight and improvement of cardiovascular risk factors (Robert et al., 2015). In a more recent study, treatment with liraglutide, combined with intensive behavioral therapy, showed greater short-term improvements in dietary disinhibition, binge eating, general eating disorder psychopathology, shape and weight concerns, compared to the intensive behavioral therapy alone (Chao et al., 2019). Another analogue of GLP-1, dulaglutide, has been investigated in BED patients with type 2 diabetes. In this pilot open label, prospective controlled study, dulaglutide treatment effectively diminished binge eating, and improved anthropometric and metabolic parameters in BED patients with type 2 diabetes (Da Porto et al., 2020). The results of these studies highlight a potential role of GLP-1 receptor agonist for the

treatment of BED, with a particular focus on subjects that suffer not only of compulsive over-eating, but also of the associated comorbid conditions, such as obesity and diabetes.

Naltrexone-bupropion (NB):

NB is a combination formulation, used for the treatment of long-term management of overweight, in adjunct to exercise and reduced-calorie diet. NB formulation was licensed for the pharmacotherapy of obesity by EMA and by the FDA in 2014 (Onakpoya et al., 2020). Functionally, naltrexone is an opioid receptors antagonist while bupropion is an antidepressant, which acts increasing dopamine levels in specific brain regions, leading to a decrease in appetite (Wang et al., 2014). The satiety effect of this formulation appears mediated by the influence on the reward system and by the bupropion stimulation of pro-opiomelanocortin (POMC) hypothalamic neurons, whose auto-inhibition is blocked by naltrexone (Billes et al., 2014; Wang et al., 2014). Considering the appetite-promoting effects of NB, and its influence on reward sensitivity, this formulation was also investigated in the management of BED. Results from two preliminary studies revealed that NB administration led to reduction of binge eating score, depression, and body weight in obese subjects with major depressive disorder and it also decreased pathological over-eating and binge eating scores in obese BED subjects (Carbone et al., 2021; Guerdjikova, Walsh, et al., 2017). Recently, a placebo-controlled double-blind pilot randomized clinical trial evaluated the effect of a fixed-dose of NB (50/300 mg) relative to placebo in obese subjects with BED. During the treatment, there was a reduction in the primary outcomes measured (binge eating, eating disorder psychopathology, depression, and body weight), even though

changes were not significantly different. The NB formulation was well tolerated in obese BED subjects (Grilo, Lydecker, et al., 2021). Subsequently, the potential effectiveness of NB has been tested in a randomized double-blind placebo-controlled trial, alone or in combination with behavioral weight loss therapy. In obese subjects with comorbid BED, both behavioral weight loss therapy and drug treatment led to an improvement in binge eating, even though no interaction of the two conditions was found (Grilo et al., 2022). Altogether, these studies provide evidence for the potential use of the NB formulation in the treatment of BED.

Phentermine-Topiramate:

The combined formulation of phentermine and topiramate extended-release (Qsymia®, Vivus, Inc.) is indicated for chronic weight management, reduced-calorie diet and physical activity in adults with a BMI of 30 kg/m^2 or greater (obesity) or 27 kg/m^2 or greater (overweight) in the presence of at least one weight-related comorbidity, such as hypertension, type 2 diabetes or dyslipidemia (Smith et al., 2013). Phentermine is a centrally acting sympathomimetic amine structurally related to amphetamine, which increases norepinephrine release, and to a lesser extent dopamine and serotonin release, suppressing appetite and food consumption. Topiramate, an antiepileptic drug with known appetite-suppressant effects, has a complex mechanism of action, comprising the blockade of voltage-gated sodium channels, an increase in gamma-aminobutyrate activity, antagonism of glutamate receptors, and inhibition of carbonic anhydrase (Halpern et al., 2013; Smith et al., 2013). The rationale for their combination is that phentermine is a sympathomimetic and a stimulant, while topiramate has sedative properties. They have opposite effects on adverse reactions,

but an additive result on weight-loss, allowing the use of lower doses (Halpern et al., 2013). Two preliminary studies of the same group highlighted the potential effectiveness of this formulation in BED. In the first study, the authors reported a successful intervention with phentermine-topiramate in two patients with BED plus obesity, who experienced cessation of binge eating behaviors and clinically significant weight-loss after the treatment (Guerdjikova et al., 2015). Subsequently, in a twelve-weeks, open-label, prospective flexible-dose trial in overweight/obese individuals with BED, phentermine-topiramate was well tolerated and effective in decreasing BMI, frequency of binge eating episodes and BED psychopathology (Guerdjikova et al., 2018). A double-blind randomized crossover trial further supported the use of phentermine-topiramate in BED (and possibly in BN), due to a significant reduction in binge-days frequency, body weight-loss and eating disorders-related psychopathology with pharmacological intervention relative to the placebo, in patients affected by BED or BN (Safer et al., 2020). These results were really promising, but their principal limit was the small number of individuals in the study. Future clinical investigations with larger sample sizes are needed to confirm the previous positive observations.

2. THE AIM OF MY STUDIES

Considering that pharmacological approaches for BED are very limited (only LDX is currently approved for this psychiatric disorder), the research for innovative and effective therapies is strictly necessary. Thus, my scientific activity has been primarily directed towards the investigation of the behavioral effects of different innovative compounds in a preclinical model of binge eating developed by Cifani et al. in 2009 (Cifani et al., 2009). In this animal model, the episode of binge eating is elicited in female rats by combining cycles of caloric restriction with a frustration stress procedure (Cifani et al., 2009). An additional aim of my research activity was to characterize this animal model from a neurobiological point of view, trying to identify which molecular changes in the central nervous system might be associated and drive to compulsive over-eating of HPF. The first topic discussed in my experimental thesis is the Sigma1 receptor (σ1R). Experimental evidence demonstrated a role for this receptor in mediating compulsive-like over-eating. Specifically, neurobiological alterations of the σ1R system have been found in animal models of binge-like eating, and σ1R antagonists are able to block excessive consumption of HPF. During my study, performed in collaboration with the medicinal chemistry unit of the University of Camerino, we have demonstrated that a very highly potent and selective σ1R antagonist, analogue of spipethiane, was effective in blocking the binge eating episode in our preclinical model. We have further characterized the behavioral effects of this compound, to analyze whether its ability to influence compulsive-like over-eating might be related to a secondary effect on anxiety- or depressive-like behaviors. Interestingly, no effect of compound'administration was observed on anxiety- and depressive-like behaviors, suggesting that antagonism of the σ1R selectively influences the neurobiological mechanisms driving compulsive-like eating behavior.

The second topic of my experimenthal thesis is focused on the role of orexin (OX) neurotransmission in binge eating behavior. OXs are hypothalamic neuropeptides with multiple behavioral and physiological functions, among which there is the control of palatable food intake. In particular, orexin-A (OX-A) is known to drive aberrant feeding patterns, including binge eating behavior. In this work, we tested the effect of the selective orexin 1 receptor antagonist (SO1RA) ACT-539313 in a prelinical model of binge eating, evaluating the efficacy of this compound under both acute and chronic administration regimen. Furthermore, I evaluated if the development of binge eating behavior was associated with changes in OX expression in the hypothalamus or in brain regions implicated in compulsive over-eating. Additionally, potential effects on the expression of delta FosB, a marker of long term neuronal adaptations and plasticity, in multiple brain regions were evaluated. These studies were conducted as part of a research collaboration agreement, and related letter agreements, between Idorsia Pharmaceuticals Ltd, Switzerland, the company where ACT-539313 was discovered and developed, and the University of Camerino, Italy, entered on Oct 15th 2021.

3. NOVEL HIGHLY POTENT AND SELECTIVE SIGMA1 RECEPTOR ANTAGONISTS EFFECTIVELY BLOCK THE BINGE EATING EPISODE IN FEMALE RATS

3.1. Introduction

Sigma receptors (σRs) are poorly understood transmembrane proteins, involved in numerous cellular functions and with multiple roles in both physiological and disease conditions in humans (Smith, 2017). They were initially identified as subtypes of the opioid receptor family. Currently, σRs are recognized as an independent receptor family, which comprises two pharmacologically distinct subtypes, the Sigma 1 receptor (σ1R) and the Sigma 2 receptor (σ2R) (Bowen, 2000; Quirion et al., 1992). They are widely distributed in the central nervous system as well as in some peripheral tissues such as gastrointestinal tract, kidney, liver, lung, heart, and adrenal medulla (Guitart et al., 2004; Rousseaux & Greene, 2016). The σ1R has been cloned from various tissues of different species (Hanner et al., 1996; Kekuda et al., 1996; Pan et al., 1998), while the σ2R was recently cloned and identified as the transmembrane protein 97 (Alon et al., 2017). The σ1R is a chaperone protein principally found within the mitochondria associated endoplasmic reticulum membranes (Hayashi & Su, 2007). It modulates Ca^{2+} signaling through the inositol trisphosphate receptor and the hippocampal dendritic spine formation, regulating levels of reactive oxygen species (Su et al., 2010; Tsai et al., 2009). Furthermore, the σ1R influences dopaminergic, glutamatergic, and cholinergic neurotransmission, and the opening of cation channels (Na^+, K^+ and Ca^{2+}) (Rousseaux & Greene, 2016). Different endogenous ligands have been proposed for the σ1R, including sphingosine (and its derivatives), progesterone, dehydroepiandrosterone, cholesterol and the hallucinogen N,N-dimethyltryptamine

(Ramachandran et al., 2009). Considering their wide distribution throughout different human tissues and their implication in many pathophysiological processes, the σ1Rs are very attractive pharmacological targets for the treatment of various diseases. Centrally active σ1R agonists demonstrated efficacy in depression, learning, delirium, akathisia and hyperkinetic movements, as well as in Alzheimer's and Parkinson's diseases (Albayrak & Hashimoto, 2013; Francardo et al., 2014; Furuse & Hashimoto, 2009; Hashimoto & Furuse, 2012; Marrazzo et al., 2005; Zvejniece et al., 2014). On the other hand, σ1R antagonists counteract neuropathic pain and they might represent useful anticancer agents (Diaz et al., 2009; Happy et al., 2015). Several studies have also revealed a role for the σ1R in drug addiction, highlighting the anti-addictive properties σ1R antagonists, able to attenuate the effects induced by both psychostimulants and ethanol (Blasio et al., 2015; Matsumoto et al., 2002; Matsumoto et al., 2008; Sabino et al., 2009). Additionally, there is experimental evidence for the involvement of the σ1R in binge eating behavior. Indeed, in 2012, a study of Cottone et al. reported that the σ1R antagonist BD-1063 (σ1R pKi = 8.05, σ1R/σ2R selectivity ratio = 71) dose-dependently decreased binge-like eating and the increased eating rate in palatable rats (Cottone et al., 2012). This was accompanied by a decrease in σ1R mRNA expression in the prefrontal and anterior cingulate cortices, and by an increase in σ1R protein expression in anterior cingulate cortex in bingeing rats, compared to the control counterpart (Cottone et al., 2012). The results of this study evidenced a role for the σ1R system in the neurobiological adaptations driving compulsive-like over-eating. Another σ1R antagonist, PD144418, proved to reduce the motivation to obtain HPF under a progressive ratio schedule of reinforcement, without any influence on appetite and food palatability (Tapia et al., 2019). Our group has also recently shown that the spipethiane analogue 2-(1-benzylpiperidin-4-yl)thiochroman-4-one

(represented in Figure 3), a potent σ1R antagonist with an outstanding σ1R/σ2R selectivity ratio of 29510, blocked the binge eating episode in female rats, further evidencing the implication of the σ1Rs in bingeing-related eating disorders (Del Bello et al., 2019).

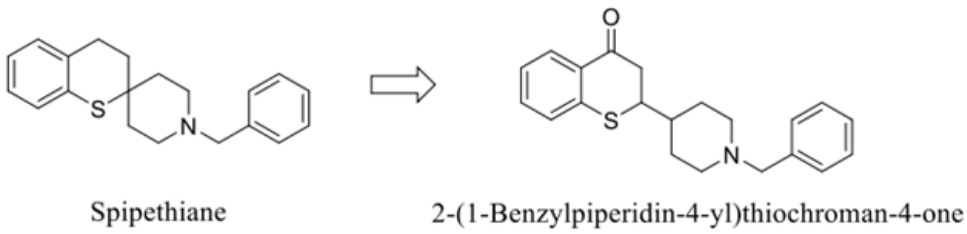


Figure 3. Chemical structures of the σ1R antagonists Spipethiane and 2-(1-benzylpiperidin-4-yl)thiochroman-4-one. Adapted from (Cifani et al., 2020).

Another potent σ1R (pKi = 10.05) and spipethiane analogue, characterized by elevated σ1R/σ2R selectivity (2515), is the 1,3-benzodioxane derivative **1** (Figure 4). Functional assays on MCF-7 and MCF-7/ADR demonstrated the σ1R antagonist activity of this compound (Piergentili et al., 2010). In this study, to improve the σ1R affinity and selectivity over the σ2R, the conformationally constrained 1,3-benzodioxane moiety of compound **1** was substituted with more flexible 1,3-dioxane nucleus, through a benzo-cracking approach. Specifically, derivatives **2** and **3**, with the phenyl substituent linked to positions 4 and 5 of the 1,3-dioxane ring, respectively, were synthesized and studied (Figure 4). To investigate the effect of the distance between the two hydrophobic portions that flank the basic function of **2** and **3**, diastereomers **4**a/b and **5**a/b were additionally synthesized. In these derivatives, the N-benzylpiperidine moiety is spaced from the 1,3-dioxane ring (Figure 4), with a further increase of the conformational flexibility of the molecule. The separation of the cis

and trans diastereomers of **4** and **5** allowed us to investigate the role of the relative configuration on σ1R affinity. The novel derivatives **2**−**5** were tested by radioligand binding assays at the σ1R and σ2R. To support the implication of the σ1R in binge eating behavior, we tested the most interesting compound **3** in Cifani et al. female rat model of binge eating (Cifani et al., 2009). Finally, affinities of compounds **2** and **3** were determined at phencyclidine (PCP) binding site of the ionotropic N-methyl-D-aspartate (NMDA) receptor, opioid receptors, and/or DAT, considering their role in BED and that different σ1R ligands also bind these targets with high affinity.

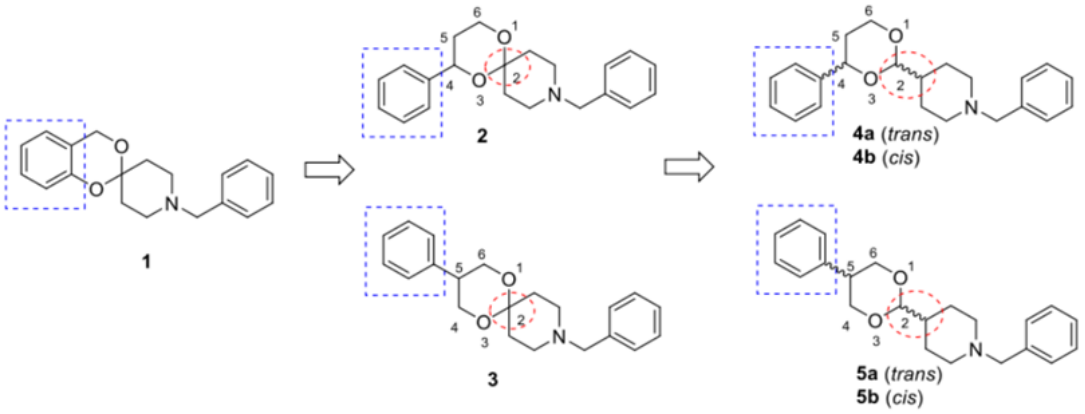


Figure 4. Chemical structures of compounds 2-5, analogues of the potent σ1R ligand 1 (Cifani et al., 2020).

3.2. Methods

3.2.1. Chemistry

*Instruments used for the synthesis and characterization of compounds **2-9***

Melting points (mp) were taken in glass capillary tubes on a Büchi SMP-20 apparatus and are uncorrected. NMR spectra were recorded on either Varian Mercury AS400 or Bruker 500 MHz instruments and chemical shifts (ppm) are reported relative to

tetramethylsilane. Spin multiplicities are given as s (singlet), d (doublet), dd (double doublet), t (triplet), or m (multiplet). IR spectra were recorded on PerkinElmer 297 instrument and spectral data (not shown because of the lack of unusual features) were obtained for all compounds reported and are consistent with the assigned structures. The microanalyses were recorded on FLASH 2000 instrument (ThermoFisher Scientific). The elemental composition of the compounds agreed to within ±0.4% of the calculated value. Mass spectra were obtained using a Hewlett Packard 1100 MSD instrument utilizing electron-spray ionization (ESI). All reactions were monitored by thin-layer chromatography (TLC) using silica gel plates (60 F254; Merck), visualizing with ultraviolet light. Chromatographic separations were performed on silica gel columns (Kieselgel 40, 0.040−0.063 mm, Merck) by flash chromatography. Compounds were named following IUPAC rules as applied by ChemBioDraw Ultra (version 11.0) software for systematically naming organic chemicals.

*9-Benzyl-2-phenyl-1,5-dioxa-9-azaspiro[5.5]undecane (**2**).*

A mixture of **8** (1.7 g, 11.16 mmol), 10 (2.11 g, 11.16 mmol), and p-toluenesulfonic acid (0.85 g, 4.85) in toluene (50 mL) was heated at reflux for 5 h. After the mixture was cooled, water was added. The aqueous phase was basified with 2 N NaOH and extracted three times with $CHCl_3$. The organic phase was dried (Na2SO4) and evaporated. The residue was purified by flash chromatography. Eluting with cyclohexane/EtOAc (7:3) afforded an oil (71% yield). ^{1}H NMR ($CDCl_3$) δ 1.61−2.60 (m, 10H, CH_2 and piperidine), 3.51 (s, 2H, NCH_2Ar), 3.90 (m, 1H, CH_2O), 4.13 (m, 1H, CH_2O), 4.98 (dd, 1H, ArCHO), 7.21−7.42 (m, 10H, ArH). ESI/MS: m/z 324.2 [M + H]+. The free base was transformed into the oxalate salt that was crystallized from

EtOH: mp 202−204 °C. Anal. Calcd for $C_{21}H_{25}NO_2 \cdot H_2C_2O_4$: C, 66.81%; H, 6.58%; N, 3.39%. Found: C, 67.05%; H, 6.42%; N, 3.50%.

*9-Benzyl-3-phenyl-1,5-dioxa-9-azaspiro[5.5]undecane (**3**).*

This compound was synthesized from **9** and **10** according to the procedure described for **2**: an oil was obtained (70% yield). ^{1}H NMR ($CDCl_3$) δ 1.82 (m, 2H, piperidine), 2.18 (m, 2H, piperidine), 2.50 (m, 4H, piperidine), 3.18 (m, 1H, CHAr), 3.53 (s, 2H, NCH_2Ar), 3.99 (m, 4H, 2 × CH_2O), 7.21−7.39 (m, 10H, ArH). ESI/MS: m/z 324.2 [M + H]+. The free base was transformed into the oxalate salt that was crystallized from EtOH: mp 211−212 °C. Anal. Calcd for $C_{21}H_{25}NO_2 \cdot H_2C_2O_4$: C, 66.81%; H, 6.58%; N, 3.39%. Found: C, 66.59%; H, 6.40%; N, 3.19%.

*1-Benzyl-4-(4-phenyl-1,3-dioxan-2-yl)piperidine (**4**).*

This compound was synthesized from **8** and **11** according to the procedure described for **2**, to give a mixture of the diastereomers **4**a and **4**b, that were separated by flash chromatography, eluting with cyclohexane/ EtOAc (95:5). The isomer **4**a eluted first as an oil (15% yield). ^{1}H NMR ($CDCl_3$) δ 1.28−2.42 (m, 9H, piperidine), 2.94 (m, 2H, piperidine), 3.49 (s, 2H, NCH_2Ar), 3.92 (m, 1H, CH_2O), 4.16 (m, 1H, CH_2O), 4.42 (d, 1H, J = 6.5 Hz, OCHO), 5.19 (m, 1H, ArCHO), 7.20−7.42 (m, 10H, ArH). ESI/MS: m/z 338.2 [M + H]+. The free base was transformed into the oxalate salt that was crystallized from 2-PrOH: mp 101−102 °C. Anal. Calcd for $C_{22}H_{27}NO_2 \cdot H_2C_2O_4$: C, 67.43%; H, 6.84%; N, 3.28%. Found: C, 67.27%, H, 6.96%; N, 3.50%. The second fraction was the isomer 4b (48% yield). ^{1}H NMR ($CDCl_3$) δ 1.19−1.94 (m, 9H, piperidine), 2.92 (m, 2H, piperidine), 3.50 (s, 2H, NCH_2Ar), 3.89 (m, 1H, CH_2O), 4.20

(m, 1H, CH_2O), 4.48 (d, 1H, J = 5.6 Hz, OCHO), 4.65 (dd, 1H, J = 11.3, 2.3 Hz, ArCHO), 7.20−7.42 (m, 10H, ArH). ESI/MS: m/z 338.2 [M + H]+. The free base was transformed into the oxalate salt that was crystallized from EtOH: mp 161−162 °C. Anal. Calcd for $C_{22}H_{27}NO_2 \cdot H_2C_2O_4$: C, 67.43%; H, 6.84%; N, 3.28%. Found: C, 67.70%, H, 6.98%; N, 3.05%.

*1-Benzyl-4-(5-phenyl-1,3-dioxan-2-yl)piperidine (**5**).*

This compound was synthesized from **9** and **11** according to the procedure described for **2**, to give a mixture of the diastereomers **5**a and **5**b, that were separated by flash chromatography eluting with cyclohexane/ EtOAc (95:5). The isomer **5**a eluted first as an oil (44% yield). ^{1}H NMR ($CDCl_3$) δ 1.40−1.98 (m, 7H, piperidine), 2.88 (m, 2H, piperidine), 3.18 (m, 1H, CHAr), 3.50 (s, 2H, NCH_2Ar), 3.78 (dd, 1H, J = 11.3, 10.8 Hz, CH_2O), 4.17 (dd, 1H, J = 11.3, 4.5 Hz, CH_2O), 4.36 (d, 1H, J = 4.9 Hz, OCHO), 7.12−7.38 (m, 10H, ArH). ESI/MS: m/z 338.2 [M + H]+. The free base was transformed into the oxalate salt that was crystallized from 2- PrOH: mp 158−160 °C. Anal. Calcd for $C_{22}H_{27}NO_2 \cdot H_2C_2O_4$: C, 67.43%; H, 6.84%; N, 3.28%. Found: C, 67.55%, H, 6.70%; N, 3.48%. The second fraction was the isomer **5**b (24% yield). ^{1}H NMR ($CDCl_3$) δ 1.42−1.97 (m, 7H, piperidine), 2.61 (m, 1H, CHAr), 2.92 (m, 2H, piperidine), 3.50 (s, 2H, NCH_2Ar), 4.18 (m, 4H, 2 × CH2O), 4.42 (d, 1H, J = 5.2 Hz, OCHO), 7.18−7.59 (m, 10H, ArH). ESI/MS: m/z 338.2 [M +H]+. The free base was transformed into the oxalate salt that was crystallized from 2-PrOH: mp 111−112 °C. Anal. Calcd for $C_{22}H_{27}NO_2 \cdot H_2C_2O_4$: C, 67.43%; H, 6.84%; N, 3.28%. Found: C, 67.61%, H, 6.97%; N, 3.41%.

*1-Phenylpropane-1,3-diol (**8**).*

A solution of **6** (Aldrich) (1 g, 4.23 mmol) in dry Et2O (3 mL) was added dropwise to a suspension of $LiAlH_4$ (0.17 g, 4.5 mmol) in dry Et2O (5 mL) at 0 °C under a nitrogen atmosphere. The mixture was stirred for 2 h at room temperature, then it was poured onto ice, and 2.5 M NaOH (12.65 mL) was added. After the precipitate was filtered off over Celite, the organic phase was dried (Na2SO4). The evaporation of the solvent afforded a residue that was purified by flash chromatography. Eluting with cycloexane/EtOAc (75:25) gave an oil (69% yield). ^{1}H NMR ($CDCl_3$) δ 1.86 (m, 2H, CH2), 3.24 (br s, 2H, exchangeable with D2O, 2 × OH), 3.79 (m, 2H, CH_2O), 4.88 (dd, 1H, CHO), 7.25−7.36 (m, 5H, ArH).

*2-Phenylpropane-1,3-diol (**9**).*

This compound was synthesized from **7** (Aldrich) according to the procedure described for 8: a white solid was obtained (72% yield). Mp 49−50 °C. ^{1}H NMR ($CDCl_3$) δ 1.95 (br s, 2H, exchangeable with D2O, 2 × OH), 3.08 (m, 1H, CHAr), 3.96−4.03 (m, 4H, 2 × CH_2O), 7.34−7.47 (m, 5H, ArH).

3.2.2. Radioligand Binding Studies at the PCP site of NMDA receptor, DAT, σ1R and σ2R and μ, κ, δ opioid receptors

Materials

The guinea pig brains, rat brains and rat liver for the σ1R, σ2R, μ-, κ- and δ-opioid receptor binding assays were commercially available (Harlan-Winkelmann, Borchen,

Germany). The pig brains for the performance of the binding assay to the PCP-binding site of the NMDA receptor were a kind donation of the local slaughterhouse (Coesfeld, Germany). Homogenizers: Elvehjem Potter (B. Braun Biotech International, Melsungen, Germany) and Soniprep 150, MSE, London, UK). Centrifuges: Cooling centrifuge model Rotina 35R (Hettich, Tuttlingen, Germany) and High-speed cooling centrifuge model Sorvall RC-5C plus (Thermo Fisher Scientific, Langenselbold, Germany). Multiplates: standard 96-well multiplates (Diagonal, Muenster, Germany). Shaker: self-made device with adjustable temperature and tumbling speed (scientific workshop of the institute). Harvester: MicroBeta FilterMate-96 Harvester. Filter: Printed Filtermat Typ A and B. Scintillator: Meltilex (Typ A or B) solid state scintillator. Scintillation analyzer: MicroBeta Trilux (all Perkin Elmer LAS, Rodgau-Jügesheim, Germany).

Preparation of membrane homogenates from pig brain cortex

Fresh pig brain cortex was homogenized with the potter (500-800 rpm, 10 up-and-down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 31,000 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of TRIS/EDTA buffer (5 mM/1 mM, pH 7.5) and centrifuged again at 31,000 x g (20 min, 4 °C). The final pellet was resuspended in 5-6 volumes of buffer and frozen (−80 °C) in 1.5 mL portions containing about 0.8 mg protein/mL.

Preparation of membrane homogenates from guinea pig brain

5 guinea pig brains were homogenized with the potter (500-800 rpm, 10 up-and-down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 23500 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 7.4) and centrifuged again at 23500 x g (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5-6 volumes of buffer and frozen (−80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

Preparation of membrane homogenates from rat brain

5 rat brains (species: Sprague Dawley rats) were homogenized with the potter (500-800 rpm, 10 up-and-down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1200 x g for 10 min at 4 °C. The supernatant was separated and centrifuged at 23500 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 7.4) and centrifuged again at 23500 x g (20 min, 4 °C). This procedure was repeated twice. The final pellet was resuspended in 5-6 volumes of buffer and frozen (−80 °C) in 1.5 mL portions containing about 1.5 mg protein/mL.

Preparation of membrane homogenates from rat liver

Two rat livers were cut into small pieces and homogenized with the potter (500-800 rpm, 10 up-and-down strokes) in 6 volumes of cold 0.32 M sucrose. The suspension was centrifuged at 1,200 x g for 10 min at 4 °C. The supernatant was separated and

centrifuged at 31,000 x g for 20 min at 4 °C. The pellet was resuspended in 5-6 volumes of buffer (50 mM TRIS, pH 8.0) and incubated at room temperature for 30 min. After the incubation, the suspension was centrifuged again at 31,000 x g for 20 min at 4 °C. The final pellet was resuspended in 5-6 volumes of buffer and stored at -80,°C in 1.5 mL portions containing about 2 mg protein/mL.

Protein determination

The protein concentration was determined by the method of Bradford,1 modified by Stoscheck.2 The Bradford solution was prepared by dissolving 5 mg of Coomassie Brilliant Blue G 250 in 2.5 mL of EtOH (95 %, v/v). 10 mL deionized H2O and 5 mL phosphoric acid (85%, m/v) were added to this solution, the mixture was stirred and filled to a total volume of 50.0 mL with deionized water. The calibration was carried out using bovine serum albumin as a standard in 9 concentrations (0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0 and 4.0 mg/mL). In a 96-well standard multiplate, 10 µL of the calibration solution or 10 µL of the membrane receptor preparation were mixed with 190 µL of the Bradford solution, respectively. After 5 min, the UV absorption of the protein-dye complex at $\lambda = 595$ nm was measured with a platereader (Tecan Genios, Tecan, Crailsheim, Germany).

General procedures for the binding assays

The test compound solutions were prepared by dissolving approximately 10 µmol (usually 2-4 mg) of test compound in DMSO so that a 10 mM stock solution was obtained. To obtain the required test solutions for the assay, the DMSO stock solution was diluted with the respective assay buffer. The filtermats were presoaked in 0.5%

aqueous polyethylenimine solution for 2 h at room temperature before use. All binding experiments were carried out in duplicates in the 96-well plates. The concentrations given are the final concentration in the assay. Generally, the assays were performed by addition of 50 µL of the respective assay buffer, 50 µL test compound solution in various concentrations (10-5, 10-6, 10-7, 10-8, 10-9 and 10-10 mol/L), 50 µL of corresponding radioligand solution and 50 µL of the respective receptor preparation into each well of the multiplate (total volume 200 µL). The receptor preparation was always added last. During the incubation, the plates were shaken at a speed of 500-600 rpm at the specified temperature. Unless otherwise noted, the assays were terminated after 120 min by rapid filtration using the harvester. During the filtration each well was washed five times with 300 µL of water. Subsequently, the filtermats were dried at 95 °C. The solid scintillator was melted on the dried filtermats at a temperature of 95 °C for 5 minutes. After solidifying of the scintillator at room temperature, the trapped radioactivity in the filtermats was measured with the scintillation analyzer. Each position on the filtermat corresponding to one well of the multiplate was measured for 5 min with the [3H]-counting protocol. The overall counting efficiency was 20%. The IC50-values were calculated with the program GraphPad Prism® 3.0 (GraphPad Software, San Diego, CA, USA) by non-linear regression analysis. Subsequently, the IC50 values were transformed into Ki-values using the equation of Cheng and Prusoff. The Ki-values are given as mean value + standard error of the mean (SEM) from three independent experiments.

Performance of the binding assays

PCP binding site of the NMDA receptor:

The assay was performed with the radioligand [3H]-(+)-MK-801 (22.0 Ci/mmol; Perkin Elmer). The thawed membrane preparation of pig brain (about 100 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [3H]-(+)-MK-801, and TRIS/EDTA buffer (5 mM/1 mM, pH 7.5) at room temperature. The non-specific binding was determined with 10 µM unlabeled (+)-MK-801. The Kd-value of (+)-MK-801 is 2.26 nM.

σ1R

The assay was performed with the radioligand [3H]-(+)-Pentazocine (22.0 Ci/mmol; Perkin Elmer). The thawed membrane preparation of guinea pig brain cortex (about 100 µg of the protein) was incubated with various concentrations of test compounds, 2 nM [3H]-(+)-Pentazocine, and TRIS buffer (50 mM, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled (+)-Pentazocine. The Kd-value of (+)-Pentazocine is 2.9 nM.

σ2R

The assays were performed with the radioligand [^{3}H]DTG (specific activity 50 Ci/mmol; ARC, St. Louis, MO, USA). The thawed membrane preparations (either membrane fragments prepared from approximately 200,000 RT-4 cells containing 150 µg protein or rat liver preparation containing 100 µg protein) were incubated with

various concentrations of the test compound, 3 nM [^{3}H]DTG and buffer containing (+)-pentazocine (500 nM (+)-pentazocine in 50 mM TRIS, pH 8.0) at 37 °C (RT-4 cell fragments) or room temperature (rat liver membranes). The non-specific binding was determined with 10 µM non-labeled DTG. The Kd values are 8.3 nM (RT-4 cells) or 17.9 nM (rat liver).

κ opioid receptor

The assay was performed with the radioligand [^{3}H]-U-69593 (55 Ci/mmol, Amersham, Little Chalfont, UK). The thawed guinea pig brain membrane preparation (about 100 µg of the protein) was incubated with various concentrations of test compounds, 1 nM [^{3}H]-U-69593, and TRIS-MgCl2-Puffer (50 mM, 8 mM MgCl2, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled U-69593. The Kd-value of U-69593 is 0.69 nM.

µ opioid receptor

The assay was performed with the radioligand [3H]-DAMGO (51 Ci/mmol, Perkin Elmer LAS). The thawed guinea pig brain membrane preparation (about 100 µg of the protein) was incubated with various concentrations of test compounds, 3 nM [3H]-DAMGO, and TRIS-MgCl2-Puffer (50 mM, 8 mM MgCl2, pH 7.4) at 37 °C. The non-specific binding was determined with 10 µM unlabeled Naloxon. The Kd-value of DAMGO is 0.57 nM.

Human μ opioid receptor

HEK293 cells stably expressing hμOR were grown in a DMEM medium, supplemented with 10% FBS, 2 mM L-glutamine, 1% penicillin-streptomycin (or antibiotic/antimycotic) and hygromycin B (50 □g/mL).4 Upon reaching 80-90% confluence, cells were harvested using pre-mixed Earle's Balanced Salt Solution (EBSS) with 5 mM EDTA (Life Technologies) and centrifuged at 3,000 rpm for 10 min at 21 °C. The supernatant was removed, and the pellet was resuspended in 10 mL hypotonic lysis buffer (5 mM MgCl2, 5 mM Tris, pH 7.4 at 4 °C) and centrifuged at 14,500 rpm (~25,000 g) for 30 min at 4 °C. The pellet was then resuspended in fresh binding buffer. A Bradford protein assay (Bio-Rad, Hercules, CA) was used to determine the protein concentration. The binding buffer was made of 50 mM Tris and 5 mM $MgCl_2$ at pH 7.4.6 The experiments were performed in presence of [^{3}H]-DAMGO (final concentration 3 nM) and 30 μg/well of membranes (final concentration). The reactions were incubated for 60 min at RT and terminated by rapid filtration through Perkin Elmer Uni-Filter-96 GF/B, presoaked for 60 min in 0.5% polyethylenimine. The non-specific binding was determined using 10 μM C-TOP or cold DAMGO. The radioligand Kd (2.94 nM) was measured via radioligand saturation experiments.

δ opioid receptor

The assay was performed with the radioligand [^{3}H]-DPDPE (69 Ci/mmol, Amersham). The thawed rat membrane preparation (about 75 μg of the protein) was incubated with various concentrations of test compounds, 3 nM [^{3}H]-DPDPE, and TRIS-$MgCl_2$-PMSF-buffer (50 mM, 8 mM $MgCl_2$, 400 μM PMSF, pH 7.4) at 37 °C.

The non-specific binding was determined with 10 μM unlabeled Morphine. The K_d-value of DPDPE is 0.65 nM.

DAT radioligand binding in rat striatum

Frozen brain striata dissected from male Sprague-Dawley rat brains (supplied in ice cold PBS buffer from BioreclamationIVT (Hicksville, NY)) were homogenized in 10-20 volumes (w/v) of modified sucrose phosphate buffer (0.32M Sucrose, 7.74 mM Na_2HPO_4, 2.26mM NaH_2PO_4 adjusted to pH 7.4 at 25 C) using a Brinkman Polytron (two cycles at setting 6 for 10 s each). The tissue was centrifuged at 20,000 rpm for 10 min at 4 OC. The pellet was suspended in cold buffer and centrifuged again. The resulting pellet was resuspended in cold buffer at a concentration of 15 mg/mL OWW (original wet weight). On test day, all test compounds were freshly diluted in 30% DMSO and 70% H_2O to a stock concentration of 1 mM or 100 μM. To assist the solubilization of free-base compounds, 10 μl of glacial acetic acid was added along with the DMSO (in place of 10 μl final H_2O volume). Each test compound was then diluted into 10 half-log serial dilutions using 30% DMSO vehicle. Radioligand competition experiments were conducted in 96-well plates containing 50 μL of diluted test compound, 300 μl of fresh binding buffer, 50 μl of radioligand diluted in binding buffer ([^{3}H]-WIN35,428: 1.5 nM final concentration; ARC, Saint Louis, MO) and 100 μl of tissue preparation (1.5 mg of brain striatum membranes per well). Aliquots of [^{3}H]-WIN35,428 solution were also quantified accurately to determine how much radioactivity was added. Non-specific binding was determined using 10 μL Indatraline and total binding was determined with 30% DMSO vehicle. The reaction was started with the addition of the tissue. All compound dilutions were tested in triplicate and the

reaction incubated for 120 min at 4 OC. The reaction was terminated by filtration through Perkin Elmer Uni-Filter-96 GF/B, presoaked for 120 min in 0.05% polyethylenimine, using a Brandel 96-Well Plates Harvester Manifold (Brandel Instruments, Gaithersburg, MD). The filters were washed 3 times with 3 mL (3 x 1 mL/well) of ice cold binding buffer. 65 μL Perkin Elmer MicroScint20 Scintillation Cocktail was added to each well and filters were counted using a Perkin Elmer MicroBeta Microplate Counter (efficiency: 32.3%). IC50 values for each compound were determined from dose-response curves and Ki values were calculated using the Cheng-Prusoff equation. Kd value for [^{3}H]- WIN35,428 (28.1 nM) was determined via separate homologous competitive binding experiments. These analyses were performed using GraphPad Prism version 6.00 for Macintosh (GraphPad Software, San Diego, CA). Ki values were determined from at least 3 independent experiments and are reported as mean ± SEM.

3.2.3. In vivo studies

Binge eating experimental procedure

One-hundred and eight female Sprague Dawley rats (Charles River, Italy), 200–225 g at the beginning of the experiments, were housed under a 12-h light/dark cycle (lights on at 8:00 a.m.), at constant temperature (20–22° C) and humidity (45–55%), and with access to food and water ad libitum for two weeks before the experiments. All experiments were carried out in accordance with the European directive 2010/63/UE governing animal welfare, and with the Italian Ministry of Health guidelines for the care and use of laboratory animals. According to the dietary schedule, the rats were given standard food pellets (4RF18, Mucedola, 2.6 kcal/g) or HPF (3.63 kcal/g)

consisting of a paste prepared by mixing Nutella (Ferrero®) chocolate cream (5.33 kcal/g; 56, 31, and 7% from carbohydrate, fat, and protein, respectively), grounded food pellets (4RF18), and water in the following w/w/w percent ratio: 52% Nutella, 33% food pellets, and 15% water. The procedure for binge eating induction was performed according to our previous studies (Cifani et al., 2009; Micioni Di Bonaventura et al., 2014; Micioni Di Bonaventura, Ubaldi, et al., 2017). Briefly, two groups of female rats were housed individually in metal cages (30 × 30 × 30 cm) and exposed (or not exposed) for 24 days to three 8-day cycles of intermittent food restriction (66% of chow intake on days 1–4 and free feeding on days 5–8 of each cycle), during which they were given access to HPF for 2 h during the light cycle, between 10:00 a.m. and 12:00 a.m. (2 h after the onset of the light cycle) on days 5–6 and 13–14 of the first two cycles (total of four exposures). Although this intermittent caloric restriction caused body weight fluctuations during the three cycles, on the test day, similar body weights were detected in all rats (Figure 5).

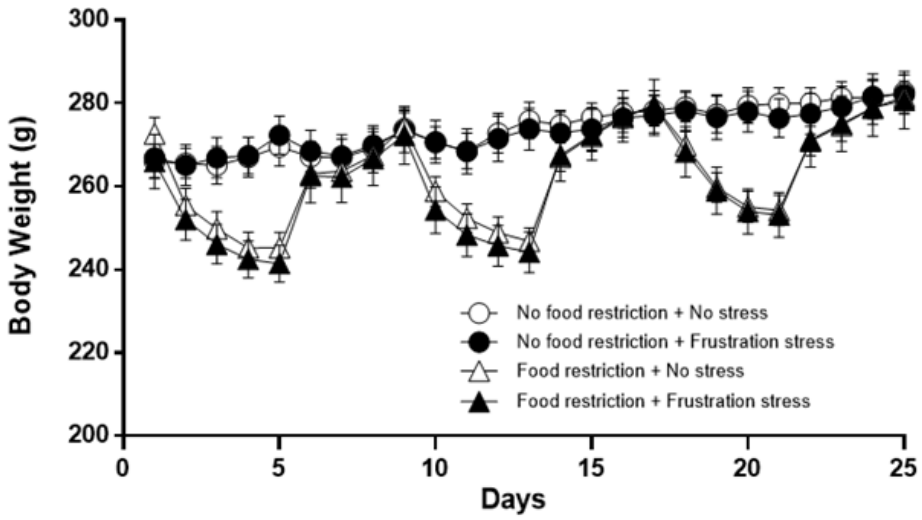


Figure 5. Mean ± SEM body weight (g) of female rats exposed or not exposed to repeated intermittent cycles of food restriction/refeeding. SEM, standard error of the mean.

On the test day (day 25), at 10 a.m., half of the rats in each group were subjected to a 15-min frustration stress, consisting of the exposure to HPF placed out of reach. During this 15-min period, the rats could smell and see the HPF and repeatedly attempted to reach it. The second half of rats in each group was not exposed to the stress manipulation. Therefore, we will refer to dietary restricted (R) vs non restricted (NR) rats and exposed to stress (S) vs non exposed to stress (NS) rats. After 15 min of stress exposure, the HPF was placed inside the cage for all rats. In accordance with our previous studies, binge eating behavior occurred in R + S rats, as demonstrated by the immediate and persistent consumption of a larger amount of HPF within the first 15-min access, with respect to the other groups. We operationally define "binge eating episode" as the significantly higher HPF consumption during the 2 h test in the R + S rats compared to the other experimental conditions. On day 25, each group was divided in three subgroups and i.p. treated with vehicle or compound 3 (at doses of 3 or 7 mg/kg), 30 min before the 2 h HPF access. Immediately after testing, we collected vaginal smears and analyzed them to assess the ovarian phase. We previously found that binge eating episode, in the present animal model, is not observed during the estrous phase. Thus, all rats in this estrous phase were excluded from the experiments (Alboni et al., 2017; Micioni Di Bonaventura, Lutz, et al., 2017).

Open field test (OFT)

OFT was performed in all groups of rats to evaluate locomotor activity, exploration, and anxiety-like behavior, measuring the following activity parameters for 10 min: total distance travelled; total vertical counts; jump counts; stereotypic counts and the number of entrances in the central zone. Automated locomotor activity boxes (square

plastic boxes with a 43 x 43 cm arena and a 25 x 25 cm central zone; Med Associates, St Albans, Vermont, USA) were used to quantify spontaneous activity parameters. Locomotor activity was recorded automatically by interruption of two orthogonal light beams (3.5 and 13 cm above the activity box floor), which were connected to automatic software. Increased locomotor activity in the entire field was considered a sign of behavioral arousal; and reduced locomotor activity in the central zone and numbers of entries into the central zone were considered signs of increased emotionality, anxiety, or fear in rodents. Between test sessions, the apparatus was cleaned with alcohol (70%) and dried with a cloth.

Forced swimming test (FST)

FST is a validated tool (Porsolt et al., 1978), used to assess the depression-like behavior in rodents inside a transparent cylinder filled with water (water temperature 23–25°C) for 5 min. The level of water was adjusted to prevent the escape of the rats and so that their tail could not touch the bottom of the cylinder. The parameter to measure, correlating with depression-like behavior, is the duration of the immobility, in which the rats merely float to keep their head above the surface of the water. The immobility observed reflected a state of lowered mood or hopelessness in animals. Each rat was subjected to two swimming sessions separated by 24 h: the first trial lasted 15 min, whereas the second one, the only monitored, 5 min. Water was changed after every trial and animals were gently dried with a towel after swimming.

Drug treatment

Compound **3** was dissolved in a 5% solution of DMSO in distilled water and i.p. administered (2 mL/kg) at 3 or 7 mg/kg doses. For the feeding test, **3** or the vehicle was injected 30 min before allowing access to the HPF.

Statistical analyses

All data were expressed as mean ± SEM. Feeding data shown in Figure 9 A, left panel were statistically analyzed by three-way ANOVA for repeated measures, which included food restriction (no, yes) and stress (no, yes) as the between-subjects factors, with sessions time (0-15, 15-30, 30-60, 60-120 min) as the within-subject factor. Feeding data shown in Figure 9 B right panel, were statistically analyzed by two-way ANOVA with food restriction and stress as the two factors. Feeding data shown in Figure 9 (B-E) were statistically analyzed by one-way ANOVA with treatment as between-subject factor. OFT and FST (Table 4) were statistically analyzed by three-way ANOVA, which included food restriction (no, yes) and stress (no, yes) and treatment (no, yes) as the between-subjects factors. We used Bonferroni's post-hoc tests to follow up on significant interaction or main effects ($P < 0.05$) from the factorial ANOVAs. We analyzed the data with Systat version 10.0 (Systat Software).

3.3. Results

3.3.1. Chemistry

The synthetic route used to synthesize derivatives **2**−**5** is illustrated in Figure 6.

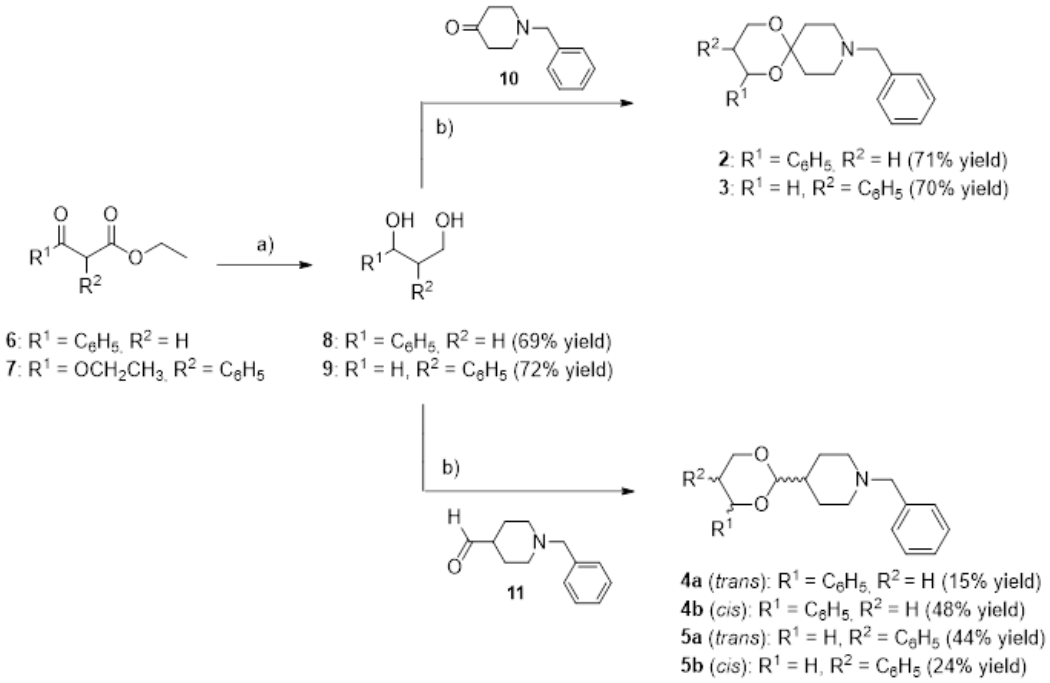
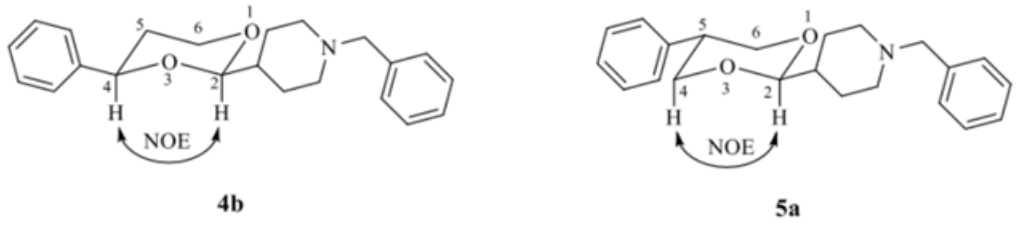


Figure 6. Schematic representation of the synthetic route used to obtain derivatives 2-5 (Cifani et al., 2020). a) LiAlH4, Et2O, r.t. for 2h b) p-toluenesulfonic acid, toluene, reflux for 5 h.

The commercially available ethyl 3-oxo-3-phenylpropionate (**6**) and diethyl 2-phenylmalonate (**7**) were subjected to a reduction reaction with $LiAlH_4$ to the corresponding diols **8** and **9**, respectively. The condensation of **8** and **9** with the suitable N-benzylpiperidine carbonyl derivatives **10** and **11** in the presence of p-toluenesulfonic acid afforded the desired derivatives **2** and **3** and the mixtures of the diastereomers **4**a/b and **5**a/b, respectively (Figure 6). The cis and trans diastereomers of **4** and **5** were separated by flash chromatography.

The stereochemical relationship between the N-benzylpiperidine moiety in position 2 and the phenyl substituent in positions 4 and 5 of **4**a/b and **5**a/b, respectively, was assessed by ^{1}H NMR analysis (NOESY studies). Specifically, an evident nuclear Overhauser effect (NOE) was found between the protons in positions 2 and 4 (4.48 and 4.65 ppm, respectively) of **4**b, evidencing that both the piperidine and phenyl rings in positions 2 and 4, respectively, are equatorially oriented. Therefore, the stereochemical relationship between the substituents in positions 2 and 4 is cis in **4**b and, consequently, trans in **4**a (Figure 7).



4b **5a**

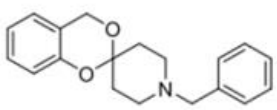
Figure 7. Structures of compounds 4b and 5a (Cifani et al., 2020).

Regarding **5**a, the axial proton in position 4 (δ 3.78 ppm) revealed two large coupling constants (J = 10.8 Hz and J = 11.3 Hz), one with the geminal equatorial proton and the other with the axial proton in position 5. Thus, the phenyl ring adopts an equatorial orientation. Furthermore, a clear NOE was found between the axial protons in positions 2 and 4 at 4.36 and 3.78 ppm, respectively, evidencing that the N-benzylpiperidine moiety also adopts an equatorial orientation. Therefore, the relative configuration between the substituents in positions 2 and 5 is trans in **5**a and, consequently, cis in **5**b (Figure 7).

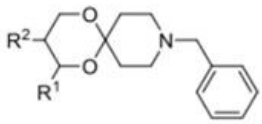
3.3.2. Radioligand Binding Studies at the PCP site of NMDA receptor, DAT, σ1R and σ2R and μ, κ, δ opioid receptors

The affinities of derivatives **2**−**5** for σ1R and σ2R were determined on guinea pig brain and rat liver membranes, respectively. [^{3}H]-(+)-pentazocine and [^{3}H]-di-o-tolylguanidine in the presence of an excess of (+)-pentazocine were used as radioligands for σ1R and σ2R, respectively (Bonifazi et al., 2015; Miyata et al., 2014). The pKi values are reported in Table 3. The lead compound **1** was included for useful comparison. Compounds **2** and **3** were additionally evaluated for their affinity for DAT, the PCP site of the NMDA receptor, as well as μ, κ, and δ opioid receptors. The assays were performed with rat striatal ([^{3}H]-WIN35,428), pig brain cortex ([^{3}H]-(+)-MK- 801), guinea pig brain ([^{3}H]-DAMGO), guinea pig brain ([^{3}H]- U-69593), and rat brain ([^{3}H]-DPDPE) membranes for DAT, NMDA, and μ, κ, and δ opioid receptors, respectively (Battiti et al., 2020; Bonifazi et al., 2015; Del Bello et al., 2018; Tangherlini et al., 2019). The pKi values are shown in Table 3.

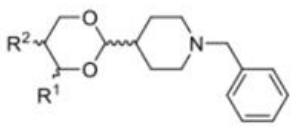
Table 3. Affinity Values (pKi) of compounds 1−5 at σ1R and σ2R and of 2 and 3 at DAT, the PCP site of the NMDA receptor, and μ, κ, and δ opioid receptors (Cifani et al., 2020).



1



2: $R^1 = C_6H_5$, $R^2 = H$
3: $R^1 = H$, $R^2 = C_6H_5$



4a (*trans*): $R^1 = C_6H_5$, $R^2 = H$
4b (*cis*): $R^1 = C_6H_5$, $R^2 = H$
5a (*trans*): $R^1 = H$, $R^2 = C_6H_5$
5b (*cis*): $R^1 = H$, $R^2 = C_6H_5$

Compd	p*K*i						
	σ1	σ2	DAT	NMDA	μ	κ	δ
1	10.05±0.08	6.65±0.09	-	-	-	-	-
2	11.00±0.07	6.33±0.11	<5	<5	<5	<5	8.60±0.14
3	10.89±0.05	6.09±0.07	5.63±0.09	<5	<5	<5	5.82±0.08
4a	8.43±0.07	6.75±0.10	-	-	-	-	-
4b	9.62±0.15	7.42±0.08	-	-	-	-	-
5a	8.44±0.14	7.25±0.02	-	-	-	-	-
5b	8.31±0.06	6.60±0.10	-	-	-	-	-

The data shown in Table 3 revealed that the benzo-cracking approach performed on the 1,3-benzodioxane derivative **1** is favorable for binding to the σ1R, while it causes a slight reduction in σ2R affinity, with a consequent increase in σ1R/σ2R selectivity. Indeed, compounds **2** and **3** have very high affinity for σ1R and remarkable σ1R/σ2R selectivity. Interestingly, compound **3** revealed an impressive σ1R/σ2R selectivity ratio (σ1R/σ2R = 63 096). A significant reduction in affinity for the σ1R and an increase in that for the σ2R are observed when the benzo-cracking approach is combined with a further increase in the distance between the two lipophilic moieties of **2** and **3** (compounds **4**a/b and **5**a/b, respectively). Consequently, the σ1R/σ2R affinity ratios of **4**a/b and **5**a/b are significantly lower than those of **2** and **3**. Stereochemistry seems to play a role in the binding to the σ1R when the phenyl ring is in position 4 of the 1,3-dioxane nucleus, with the cis isomer **4**b showing an affinity

value significantly higher than that of the trans diastereomer **4**a. On the other hand, the trans and cis 5-phenyl diastereomers **5**a and **5**b have similar affinity values. From results obtained with the off-targets, it emerges that ligand **2** shows negligible affinity for DAT, NMDA, and μ and κ opioid receptors and high affinity for the δ subtype (pK_i = 8.60), although it is 251-fold lower than that for σ1R. Interestingly, compound **3**, which also binds the δ receptor with sub-micromolar affinity, shows a remarkable selectivity for the σ1R over all the evaluated targets (σ1R/DAT = 181970, σ1R/NMDA > 776247, σ1R/μ > 776247, σ1R/κ > 776247, σ1R/δ = 117490). To rationalize the affinity profiles of the proposed ligands at the σ1R, docking simulations were performed based on the resolved σ1R structure (PDB ID: 5HK1) using the PLANTS software and following the same recently reported computational protocol (Del Bello et al., 2019). As discussed below, the complex stability is evaluated by calculating the APBS score which is focused on the polar interactions (Ren et al., 2012). Figure 8 compares the computed putative poses for **1** (Figure 8 A) and **2** (Figure 8 B) and reveals some differences which can justify the increase of affinity observed for the latter.

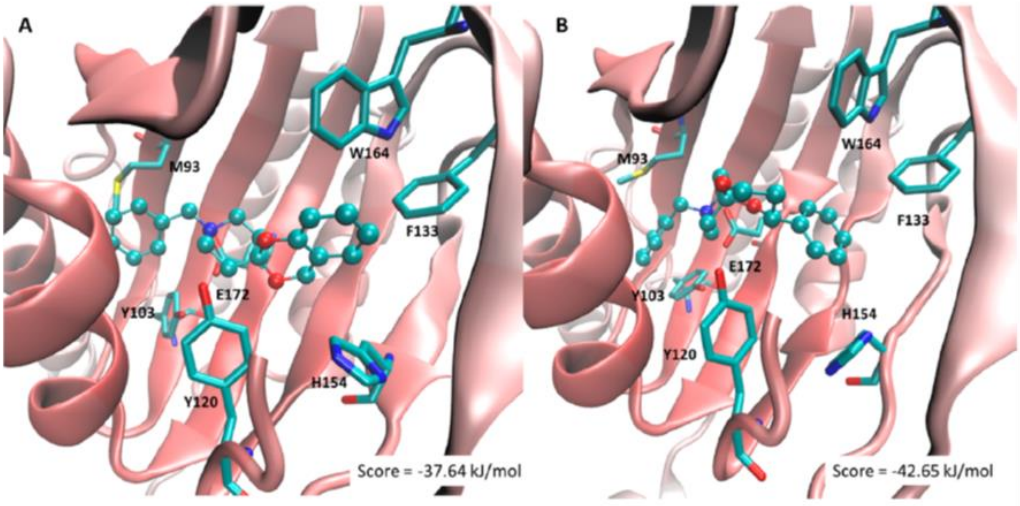


Figure 8. Main interactions stabilizing the putative complexes for 1 (A) and (R)-2 (B) as computed using the resolved σ1R structure. The reported scores are calculated by using the APBS method (Cifani et al., 2020).

In detail, Figure 8 A highlights the key interactions stabilized by **1** which can be schematized as follows: (a) the ligand ammonium head stabilizes a clear ion-pair with Glu172 reinforced by a H-bond with Tyr103; (b) the benzyl moiety is inserted within a hydrophobic subpocket where it mostly contacts alkyl side-chains plus π−π stacking with Tyr103 and a π-sulfur contact with Met93; (c) the benzodioxane system is accommodated within a subpocket lined by several aromatic residues while the O1 oxygen atom is engaged by a H-bond with Tyr120. The enantiomers of **2** afford very similar putative complexes, and attention is here focused on the complex for (R)- 2 since it shows a slightly better APBS score compared to (S)-2 (−42.56 vs −41.38 kJ/mol). Specifically, Figure 8 B emphasizes that (R)-2 elicits an interaction pattern very similar to that already seen in Figure 8 A, even though some key interactions appear to be enhanced when compared to those elicited by **1**. This positive effect can be seen in the contacts stabilized by (a) the benzyl moiety which elicits an optimized π−π stacking with Tyr103; (b) the dioxane oxygen atoms which better approach

Tyr120; and (c) the phenyl ring which is engaged by an extended set of π–π stacking interactions with Phe107, Phe133, His154, and Trp164. These reinforced contacts are reflected into better complex stability as encoded by the scores displayed in Figure 8 A. As also confirmed by its APBS score (−38.91 kJ/ mol), compound 3 yields an in-between docking result, with the two aromatic rings being engaged by enhanced contacts, while the dioxane ring is unable to conveniently approach Tyr120, as seen in Figure 8 B. Finally, compounds **4** and **5** reveal computed poses rather similar to those observed for the previous ligands, even though the free dioxane ring assumes a rather different arrangement which hampers its interactions with Tyr120. The lack of this contact induces flipped poses of the most hindered ligands by which the dioxane ring approaches Tyr103.

3.3.3. In vivo studies

Establishment of binge-like eating behavior

Compound **3** was selected for the in vivo evaluation, in a validated female rat model of binge eating (Cifani et al., 2009; Micioni Di Bonaventura et al., 2014; Romano et al., 2020). In this animal model, female rats were randomly divided into four experimental groups: non restricted and not exposed to stress group (NR + NS); non restricted and exposed to stress group (NR + S); restricted and not exposed to stress group (R + NS); restricted and exposed to stress group (R + S). In accordance with previous studies (Micioni Di Bonaventura et al., 2013; Pucci et al., 2016) the ANOVA in the vehicle groups revealed a marked interaction among the three factors (food restriction × stress × session time) [$F_{interaction}$ (3,72) = 4.8; $P < 0.01$]. Bonferroni post-hoc tests revealed a significant ($P < 0.01$) increase in HPF consumption in the first 15

min in the R + S group (bingeing group), compared to the other three groups. On the other hand, during the time of the other sessions (15−30, 30−60, 60−120 min), no change in HPF intake was observed among all groups (Figure 9 A, left panel). At the end of the binge eating test (120 min), two-way ANOVA showed a two-way interaction (food restriction × stress) [$F_{interaction}$ (1,24) = 4.3; $P < 0.05$] and the post-hoc analyses ($P < 0.01$) revealed that only R + S rats significantly enhanced HPF consumption with respect to the other groups (Figure 9 A, right panel).

Effect of compound 3 administration on binge-like eating behavior

Acute i.p. injection of compound **3**, 30 min before giving access to the HPF, selectively blocked the episode of binge eating in a dose-dependent manner in the R + S group, without affecting consumption in the other experimental groups during 120 min of observation (Figure 8 B-E). Specifically, in R + S rats, the ANOVA reported a significant effect of treatment at 0−15 min [$F(2,20) = 6.7$; $P < 0.05$] and at 0−120 min [$F(2,20) = 10.9$; $P < 0.01$]. Post-hoc analyses indicated that both doses tested (3 or 7 mg/kg) significantly decreased HPF consumption in R + S at each time point as indicated in Figure 9 E.

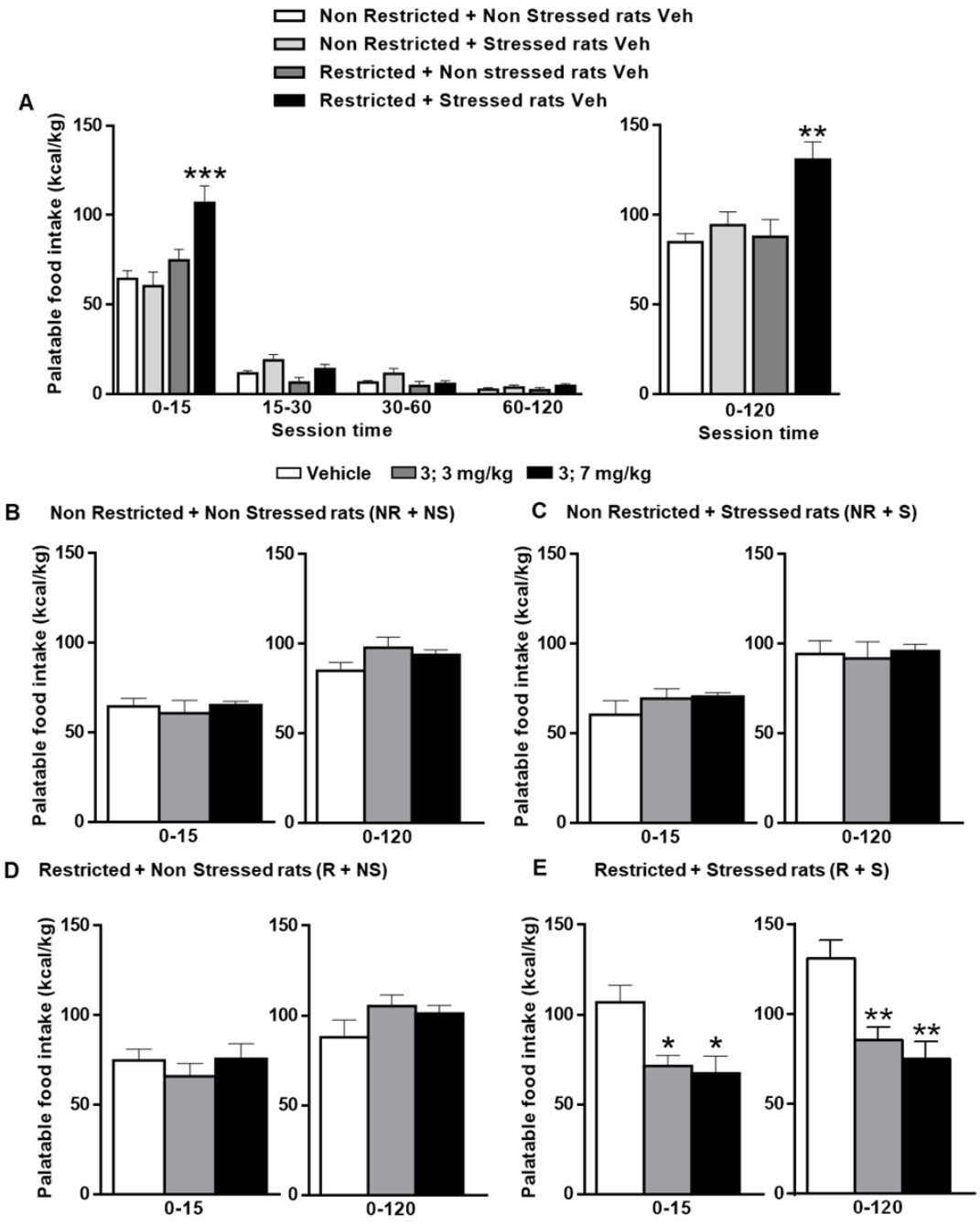


Figure 9. Establishment of binge-like eating behavior and effect of compound 3 administration on binge eating episode (Cifani et al., 2020). (A) HPF intake shown in kcal/kg at different sessions time (0−15, 15−30, 30−60, 60−120 min; left) and at 120 min (right) in the vehicle (veh) injected rats. **P < 0.01; ***P < 0.001 different from the other three groups. HPF eating (kcal/kg) after 15 min (left) or 120 min (right) to free access to cup containing chocolate paste in veh or treated rats: NR + NS (B, Non Restricted + Non Stressed), NR + S (C, Non Restricted + Stressed), R + NS (D, restricted + Non

Stressed), R + S (E, Restricted + Stressed) groups. *P < 0.05; ***P < 0.01 vs R + S veh. Data are expressed as mean ± SEM, N = 6−8 per group. SEM, standard error of the mean.

Effect of compound 3 administration in the OFT

The administration of compound **3** did not affect any measured parameters in the OFT. Indeed, analyzing the locomotor activity in the entire OFT arena, ANOVA showed a significant effect of restriction and stress conditions [$F_{restriction}$ (1,48) = 7.9; P < 0.01; F_{stress} (1,48) = 30.9; P < 0.001] and no effect of the treatment with 3 [$F_{treatment}$ (1,48) = 0.6; P > 0.05]. R + S veh and R + S **3** (7 mg/kg) groups revealed the highest distance traveled compared to the other groups (Table 4). Regarding the other parameters, jump and total vertical counts were significantly affected only by stress [F_{stress} (1,48) = 11.9; P < 0.01] and [F_{stress} (1,48) = 86.7; P < 0.001], respectively, but not by restriction or treatment conditions. As shown in Table 4, the stress procedure appeared to increase the general arousal and this effect was confirmed by the significant gain in vehicle or treated stressed rats (NR + S and R + S) on distance traveled in the central zone [F_{stress} (1,48) = 19.7; P < 0.001] and on zone entries [F_{stress} (1,48) = 39.2; P < 0.001] into the central zone. No difference in the stereotypic counts was found among the groups [$F_{restriction}$ (1,48) = 0,7; P > 0.05; F_{stress} (1,48) = 0.02; P > 0.05; $F_{treatment}$ (1,48) = 1.6; P > 0.05].

Effect of compound 3 administration in the FST

Using the FST, the ANOVA revealed that the immobility time was significantly impacted by restriction [$F_{restriction}$ (1,49) = 8,2; P < 0.01], stress [F_{stress} (1,49) = 11.5; P < 0.01] and by the interaction of these two factors [$F_{interaction}$ (1,49) = 12.7, P < 0.01],

while compound **3** [$F_{treatment}$ (1,48) = 1.6; P > 0.05] did not change the swimming/floating behavior. Post-hoc tests exhibited a significantly longer immobility time in vehicle or treated R + S rats compared with the other groups (Table 4), revealing that the cycles of food restriction plus stress may increase depression-like behavior in female rats.

Table 4. Behavioral Parameters in female rats performing the OFT and FST (Cifani et al., 2020).

OPEN FIELD TEST								
Parameters	**NR + NS**		**NR + S**		**R + NS**		**R + S**	
	Veh	**3 (7 mg/kg)**	**Veh**	**3 (7 mg/kg)**	**Veh**	**3 (7 mg/kg)**	**Veh**	**3 (7 mg/kg)**
Tot. dist. trav. (cm)	2305.3±357.3	2492.3±225.3	3602.9±269.3	3467.2±411.8	2626.2±242.5	3132.4±331.6	4335.9±419.3*	4461.5±346.3*
Tot. vert. counts	99±4	93.7±5	123.9±6.1	125.9±5.4	87±3.3	94.3±6.6	131.7±4.3	126.6±4.5
Jump counts	104.6±4.8	113±17.1	137.1±2.1	152±32.5	97.7±6.3	119.5±9.1	172±20.6	170.4±19
Stereot. counts	2367.1±116.9	2500.3±89.3	2477.4±161.4	2270.4±137.5	2030.5±280.8	2516.7±79.2	2305.1±79.7	2401±119.6
Cent. dist. trav. (cm)	101.2±25.6	112.7±7.1	140.3±30.1	148.1±13.1	84.3±17.1	96.4±6	179.9±24.8	177.5±37.5
Cent. zone entries	26.4±4.6	28.1±2.7	34.5±4.1	34±6.8	18.7±2.8	22.5±2.1	50.4±2.4	47.2±10
FORCED SWIMMING TEST								
Parameters	**NR + NS**		**NR + S**		**R + NS**		**R + S**	
	Veh	**3 (7 mg/kg)**	**Veh**	**3 (7 mg/kg)**	**Veh**	**3 (7 mg/kg)**	**Veh**	**3 (7 mg/kg)**
Immobility time (s)	100.4±12.94	106.7±11.2	92.9±13.5	111.1±11.2	99.6±10.4	94.8±9.2	158.6±10*	163.5±22.5*

In the entire open field arena: tot. dist. trav. (cm), total distance traveled; tot. vert. counts, total vertical counts; jump counts; stereot. counts, stereotypic counts. In the central zone of the open field box: cent. dist. trav. (cm), distance travelled in the center; cent. zone entries, number of entrances in the central zone. Data are the mean ± SEM. *p < 0.05 vs the other groups. N = 6−8 per group.

3.4. Discussion

In this study, we have reported the synthesis, the biological and pharmacological characterization of a highly potent and selective σ1R antagonist, which might represent a useful pharmacological tool for the management of binge-like eating behavior.

Previously, we have demonstrated that a σ1R antagonist, analogue of spipethiane, was able to block the episode of binge eating in female rats exposed to three 8-day cycles of food restriction and re-feeding plus a frustration stress procedure (Del Bello et al., 2019). The aim of this work was to improve the σ1R affinity and selectivity profile over the σ2R subtype of another σ1R ligand and spipethiane analogue, the compound **1** (Figure 4). To do this, the benzo-cracking approach was applied to the conformationally constrained 1,3-benzodioxane structure of **1**, replacing it with the more flexible 1,3-dioxane nucleus. This led to derivatives **2** and **3**. Furthermore, the influence of the distance between the two hydrophobic portions that flank the basic function of **2** and **3** was evaluated, synthesizing the diastereomers **4**a/b and **5**a/b (Figure 4). Radioligand binding assays performed on these compounds demonstrated that the benzo-cracking approach performed on the 1,3-benzodioxane derivative **1** was favorable for binding to the σ1R, while causing a slight reduction in σ2R affinity, with a consequent increase in σ1R/σ2R selectivity. Indeed, both compounds **2** and **3** displayed very high affinity for σ1R and remarkable σ1R/σ2R selectivity. Several potent σ1R ligands belonging to different chemical classes and highly selective over the σ2R have been discovered (Arena et al., 2018). Intriguingly, compound **3** reported an impressive σ1R/σ2R selectivity ratio (σ1R/σ2R = 63 096), and it is the most selective σ1R ligand reported so far, to our knowledge. When the benzo-cracking approach was combined with a further increase in the distance between the two lipophilic moieties of **2** and **3** (compounds **4**a/b and **5**a/b, respectively), a significant reduction in the affinity for the σ1R and an increase in that for the σ2R were observed. Thus, the σ1R/σ2R affinity ratios displayed by **4**a/b and **5**a/b were significantly lower than those of compounds **2** and **3**. It is possible that the increased conformational freedom and distance between the two lipophilic portions might be detrimental for the optimal interaction with the σ1R. Interestingly, compound

3 also reported a remarkable selectivity for the σ1R over all the evaluated targets (σ1R/DAT = 181970, σ1R/NMDA > 776247, σ1R/μ > 776247, σ1R/κ > 776247, σ1R/δ = 117490) and its binding profile is noticeable, considering that several potent σ1R ligands also bind to DAT, NMDA, and/or opioid receptors with elevated affinity (Cao et al., 2008; Hiranita et al., 2011; Smith, 2017). In light of the impressive σ1R affinity and selectivity profile, compound **3** was selected for the in vivo evaluation, in a preclinical model of binge eating, in which the compulsive consumption of HPF is elicited by combining cycles of caloric restriction and stress (Cifani et al., 2009). Female rats are used considering the higher prevalence of BED and BN in women rather than in men (Hudson et al., 2007). As shown in previous studies (Cifani et al., 2009; Micioni Di Bonaventura et al., 2014; Micioni Di Bonaventura, Lutz, et al., 2017; Romano et al., 2020), the association of three consecutive food restriction/refeeding periods and acute stress triggers a significant increase in HPF intake only in R + S rats. Stress is induced by placing a coffee cup containing HPF for 15 min, letting the animal see the cup and smell the HPF odor, without the possibility to eat it. This stressful condition, although being mild, has been shown to enhance the corticosterone blood levels in stressed rats (Cifani et al., 2013; Cifani et al., 2010; Micioni Di Bonaventura et al., 2020; Micioni Di Bonaventura et al., 2012). Interestingly, administration of compound **3** was able to dose-dependently block the binge eating episode selectively in R + S rats, without any effect on HPF intake in the other groups. This finding is in accordance with previous studies showing that σ1R antagonists have the ability to prevent compulsive-like eating and motivation to obtain palatable food (Cottone et al., 2012; Del Bello et al., 2019; Tapia et al., 2019). Additionally, in order to determine whether systemic injection of compound **3** might affect different aspects of animal behavior in the control or bingeing group, the OFT and FST were performed. The OFT is a validated test, commonly used

to evaluate locomotor activity and anxiety-like behavior in rodents in an unfamiliar environment (Prut & Belzung, 2003), while the FST is a suitable tool for evaluating depressive-like behavior (Porsolt et al., 1978). However, administration of compound **3** did not affect any measured behavioral parameters in these present tests. Interestingly, in the OFT, R + S vehicle- and **3**-treated rats revealed the highest distance traveled compared to the other experimental groups. The stress procedure appeared to increase the general arousal of rodents, effect confirmed by the significant gain in vehicle or treated stressed rats (NR + S and R + S) on distance traveled in the central zone, and on entries into the central part of the OF arena. The latest findings suggest that stress does not influence anxiety-like behavior in stressed rats. Indeed, a reduction of distance traveled or low numbers of entries into the central zone of the OFT arena are signs of enhanced emotionality and anxiety in rodents (Royce, 1977). In the FST, a significantly longer immobility time was observed in vehicle or treated R + S rats compared with the other groups, indicating that the cycles of food restriction plus stress might lead to depression-like behavior in female rats. This is in accordance with human studies, in which a high degree of comorbidity between BED, depression, and depression-related disorders has been observed (Lydecker & Grilo, 2021; Udo & Grilo, 2019).

To summarize, this study evidence that the compound **3**, a very highly potent and selective σ1R antagonist, was able to block over-eating for HPF selectively in bingeing rats, without affecting the HPF intake in the control groups and the anxiety-like and depression-related behaviors in all the groups considered. This finding supports the potential use of σ1R antagonists in the treatment of bingeing-related eating disorders, pathological conditions for which current pharmacological approaches are unfortunately very limited.

4. EXPRESSION OF OREXIN-A AND DELTA FOSB IN MULTIPLE BRAIN REGIONS OF BINGE EATING RATS CHRONICALLY TREATED WITH A SELECTIVE OREXIN 1 RECEPTOR ANTAGONIST

4.1. Introduction

Orexins (OXs) are hypothalamic neuropeptides, derived from the same precursor (prepro-orexin) by proteolytic processing. These peptides are known as orexin-A (OX-A, 33 amino-acids) and orexin-B (OX-B, 28 amino-acids), and they are 46 % identical in their sequence (Sakurai et al., 1998). OX-A and OX-B act binding two closely related GPCRs, termed the orexin-1 receptor (OX1R) and orexin-2 receptor (OX2R), with different binding affinities. OX-A binds with similar affinity both the OX1R and OX2R, while OX-B binds almost exclusively the OX2R, with roughly the same affinity for this receptor as OX-A (Sakurai et al., 1998). OXs-expressing neurons are predominantly located in the hypothalamus, particularly in the lateral hypothalamic area (LHA), perifornical area (PeF), dorsomedial hypothalamus (DMH) and posterior hypothalamus (Cutler et al., 1999; de Lecea et al., 1998; Nambu et al., 1999; Peyron et al., 1998). Despite being a relatively small number of neurons confined in the hypothalamus, OX-expressing cell bodies send neural projections to multiple brain regions throughout the central nervous system. These include the brainstem (mostly the locus coeruleus (LC) and the tuberomammillary nucleus), dorsal raphe nucleus (DRN), PFC, VTA, thalamus and limbic system (Cutler et al., 1999; de Lecea et al., 1998; Nambu et al., 1999; Peyron et al., 1998). Expression of OXRs has been detected within each of these regions (Marcus et al., 2001; Trivedi et al., 1998). The wide anatomical distribution of OXs-positive fibers and OXRs indicates an important role for OX neurotransmission in numerous behavioral and physiological functions, including the regulation of sleep-

wake cycle (Cao & Guilleminault, 2011; Mignot et al., 2021), food intake (Barson, 2020), reward- and motivation-related behaviors (Barson & Leibowitz, 2017; Matzeu & Martin-Fardon, 2021) and stress response (Sargin, 2019) (Figure 9).

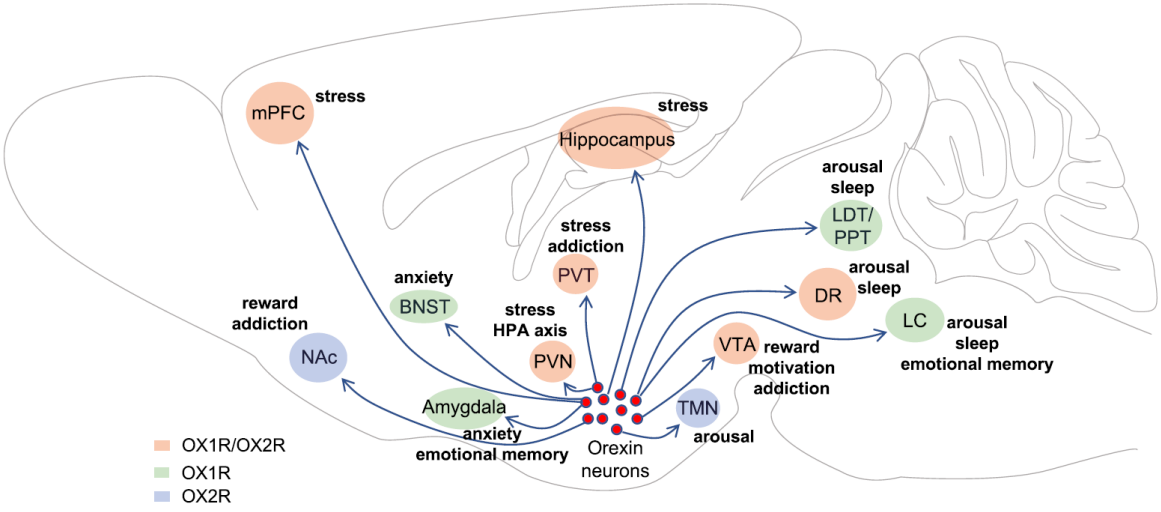


Figure 9. Representation of OXs-expressing neurons efferent projections, distribution of OXRs throughout the central nervous system and their associated behavioral and physiological functions (Sargin, 2019). BNST, bed nucleus of the stria terminalis; DR, dorsal raphe; LDT, latero-dorsal tegmental nucleus; LC, locus coeruleus; mPFC, medial prefrontal cortex; OX, orexin; OX1R, orexin-1 receptor; OX2R, orexin-2 receptor; PPT, pedunculopontine tegmental nucleus; PVN, paraventricular nucleus of the hypothalamus; PVT, paraventricular nucleus of the thalamus; TMN, tuberomammillary nucleus; VTA, ventral tegmental area.

Thus, the OX neurotransmission represents a highly attractive target for the development of medications for several psychiatric diseases. Recently, Daridorexant, an orally administered dual OXRs antagonist (Roch et al., 2021), developed by Idorsia Pharmaceuticals Ltd, has been approved for the treatment of adult patients with

insomnia characterized by difficulties in sleep onset and/or sleep maintenance (Markham, 2022).

A variety of evidence suggests a role for OXs neurotransmission in the regulation of food intake under certain conditions. Central administration of OX-A was reported to stimulate food consumption in rats, and the gene expression and peptide levels of OXs were up-regulated upon fasting (Cai et al., 1999; Fujiki et al., 2001; Sakurai et al., 1998). Additionally, under restricted feeding regimens, OX-A-expressing neurons are more active during food anticipatory periods (Johnstone et al., 2006; Mieda et al., 2004). This indicates that OXs-neurons fire with the expectation of food availability, while they are rapidly depressed at the onset of the act of eating (Gonzalez et al., 2016). The influence of OXs on the promotion of feeding predominantly occurs via OX-A and the OX1R, with minimal effects associated to OX-B (Dube et al., 1999; Lubkin & Stricker-Krongrad, 1998). In this context, OX1R antagonists, such as SB-334867, were demonstrated able to inhibit chow feeding (both natural and fasting-induced) after systemic (Haynes et al., 2000; Ishii et al., 2005) or central injections (Kay et al., 2014; Mayannavar et al., 2016). SB-334867, however, is not fully devoid of off-target effects (Gotter et al., 2012; Porter et al., 2001), thus experiments with this compound have to be regarded with caution.

However, in addition to its potential regulatory role on regular food ingestion, the OX system seems particularly able to facilitate the over-consumption and binge-like intake of HPF. Hypothalamic OX mRNA levels are up-regulated after binge-like eating of a highly palatable sugar diet (Olszewski et al., 2009) and OX-producing neurons are activated by both context-conditioned expectation and acute consumption of palatable food (Choi et al., 2010; Valdivia et al., 2015; Valdivia et al., 2014). OX-A has been demonstrated to drive compulsive-like eating even at extra-hypothalamic sites. For

example, OX-A stimulated chocolate consumption when directly injected in the Nucleus Accumbens Shell (NAcSh) (Castro et al., 2016), hedonic feeding of a high fat diet or sucrose when injected in the VTA (Terrill et al., 2016), sucrose consumption when injected in the paraventricular thalamic nucleus (PVT) (Barson et al., 2015), and palatable high fat food intake when injected in the central amygdala (CeA) (Jin et al., 2020). Interestingly, OX1R antagonist administration is effective in reducing binge-like eating in rats and mice (Alcaraz-Iborra et al., 2014; Olney et al., 2015; Piccoli et al., 2012; Valdivia et al., 2014), at doses lower than those required to inhibit chow feeding. Considering the role of OX-A and of the OX1R in compulsive palatable food intake, the aim of the present study was to investigate potential adaptations in OX-A neurotransmission in the brain of rats exposed to a well validated preclinical model of binge eating behavior (Cifani et al., 2009), in which our group has already observed that administration of a SO1RA was effective in blocking the binge eating episode in female rats (Piccoli et al., 2012). Specifically, in the current investigation, the OX-A protein expression was analyzed in rats chronically exposed to this binge eating protocol and chronically treated with a potent and selective SO1RA, named ACT-539313, provided by the pharmaceutical company Idorsia Pharmaceuticals Ltd. under a research collaboration agreement with the University of Camerino. The expression of OX-A was evaluated at extra-hypothalamic brain regions to which OX-expressing neurons project, and in the hypothalamus, were OX neurons reside. An additional aim of this neuroimaging analysis was to determine the protein expression of delta FosB, a marker of long term neuronal adaptations and plasticity (Nestler et al., 1999), in nuclei of brain regions implicated in binge eating behaviors. Delta FosB is part of the Fos family of transcription factors, but differently from the other members, like c-Fos, it is highly stable and accumulates and persists in neurons for longer periods after repeated

stimulations (Nestler, 2015; Nestler et al., 2001), including administrations of drugs of abuse (Brenhouse & Stellar, 2006; Perrotti et al., 2008), anti-depressant and anti-psychotic treatments (Dietz et al., 2014; Nestler, 2015), and stress exposure (Perrotti et al., 2004; Wang et al., 2016). Interestingly, overexpression of delta FosB in the NAc facilitates sucrose intake (Wallace et al., 2008) and food-reinforced instrumental behavior (Olausson et al., 2006). Delta FosB neuronal induction has been reported in response to caloric restriction (Vialou et al., 2011), chronic consumption of a high-fat diet (Teegarden & Bale, 2007) and sucrose (Wallace et al., 2008), and in reward-related brain regions of rats after binge-like consumption of palatable food (Munoz-Escobar et al., 2019; Quansah Amissah et al., 2020). Lastly, to evaluate a potential interaction of OX-A and delta FosB in driving compulsive-like eating behavior, the percentage of hypothalamic OX-A-expressing neurons that co-express delta FosB was also analyzed.

4.2. Methods

This study was conducted in under a research collaboration agreement with the pharmaceutical company Idorsia Pharmaceuticals Ltd, Switzerland, which provided the SO1RA ACT-539313. The methods and results described herein are filed in the proprietary Idorsia internal reports B-21.047 and B-22.042.

4.2.1. Animals

A total of N=28 female Sprague Dawley rats (Charles River; total n=28; 225–250 g at the beginning of the experiments) were used. Rats were housed under a 12 h light/dark cycle (lights on at 8:00 A.M.) with access to food and water ad libitum for 2 weeks before the experiments. They were kept in a room at constant temperature (20 –22°C)

and humidity (45–55%). All procedures were conducted in adherence to the European Community Directive for Care and Use of Laboratory Animals.

4.2.2. Diets

The rats were given standard rat food pellets (4RF18; Mucedola; 2.6 kcal/g). The highly palatable food (3.63 kcal/g) was a paste prepared by mixing Nutella (Ferrero) chocolate cream (5.33 kcal/g; 56, 31, and 7% from carbohydrate, fat, and protein, respectively), ground food pellets (4RF18), and water in the following w/w percent ratio: 52% Nutella, 33% food pellets, and 15% water. The palatable food diet was offered in a coffee cup.

4.2.3. The stress procedure

The frustration stress manipulation consisted of 15 min exposure to the coffee cup containing the palatable food that was placed inside a metallic grid container and hung up on the anterior wall of the cage. The rats could see and smell the palatable food but could not access it. During this 15 min period, the rats engaged in repeated movements of the forepaws, head, and trunk, suggesting that they were attempting to reach the palatable food. The rats were exposed (or not exposed) to the stress manipulation between 10:00 A.M. and 12:00 P.M. After 15 min, the palatable food cup was placed inside the cage, and food intake was determined for 2 h during the test conditions. The rats exposed to stress were acclimated in a room different from that of the groups not exposed to stress.

4.2.4. Experimental design

The animals were divided into 2 groups (N=14 each), namely the non-restricted and non-exposed to stress (NR + NS) group and the restricted and exposed to stress (R + S) group (the binge eating group). The rats were exposed (R + S) or not (NR + NS) for 24 days to three 8-day cycles of food restriction (66% of chow intake on days 1–4 and free feeding on days 5–8 of each cycle) during which all rats were given access to the palatable food for 2 h during the light cycle between 10:00 A.M. and 12:00 P.M. on days 5–6 and 13–14 of the first two cycles (total of four exposures). During the binge intake test (day 25), we assessed palatable food intake for 2h immediately after exposure (R + S) or not (NR + NS) to the 15-min lasting frustration stress procedure (Cifani et al., 2009). Once assigned to one of these groups, the rats remained in that group throughout the study. On day 25, the R + S and NR + NS groups were divided into 2 subgroups and orally treated with either vehicle or the SO1RA ACT-539313 at 15 mg/kg 1h before the binge eating test. The dose of ACT-539313 was selected from a previous experiment performed in the same animal model, in which it was reported to selectively attenuate the HPF intake in R + S rats, without any influence in the other experimental groups (data not shown). After one day off at the end of the first test, the two groups (NR + NS, and R + S, each vehicle and ACT-539313 treated) received an additional 8-day cycle: the two NR + NS groups had 8 days of chow ad libitum, whereas the two R + S groups had 4 days chow restricted to 66% of the normal intake followed by 4 days of chow ad libitum. In this additional cycle, no group had access to HPF, and daily treatment was continued. The following day (day 35), the R + S group was re-exposed to a 15-min lasting frustration stress while the NR + NS group was not. All rats were treated again with either vehicle or ACT-539313 15 mg/kg 1h before re-testing on day 35.

4.2.5. Drugs and formulations

On days 25 and 35, ACT-539313 or the respective vehicle were administered 1h before the exposure to the HPF by oral gavage. At the days in between, days 26-34, ACT-539313 was likewise given once daily by oral gavage at roughly a similar time of day as during testing on days 25 and 35. The vehicle of ACT-539313 was an aqueous solution of 0.5% methylcellulose (Methocel® MC, Fluka/Sigma-Aldrich, Steinheim, Germany). Drug formulations were prepared freshly at the day of administration according to a separate protocol provided by Idorsia Pharmaceuticals.

4.2.6. Animals' perfusion

The chronically vehicle- and ACT-539313-treated NR + NS and R + S rats (n = 7 per treatment and condition; n = 28 total) were euthanized about 24 h after the last HPF intake test and treatment on Day 35 (i.e., on Day 36). Rats did not receive treatment on the day of euthanasia. The exact times of euthanasia and estrous cycle were recorded for each rat. Treatment groups and conditions were randomized across the whole time period needed to perform the euthanasia and tissue collection of the 28 rats. Following terminal anesthesia with isoflurane (80 s), rats were perfused transcardially with 100 ml of 0.1 M phosphate-buffered saline (PBS), followed by 400 ml of 4% paraformaldehyde (PFA) in 0.1 M sodium phosphate, pH 7.4. Thereafter, the brains were removed from the skull and post-fixed by immersion in 4% PFA in 0.1 M PBS for 24 h at 4 °C. Finally, the brains were transferred to individual 50 ml Falcon tubes filled to the brim with PBS containing 0.001% sodium azide, and shipped to Neuroscience Associates (Knoxville, TN, USA) at ambient temperature. The brains were examined, and the left hemisphere separated by cutting the brain in half about 1 mm to the right of the midline. The

remaining right hemisphere was discarded. The olfactory bulbs of the left hemisphere were dissected and discarded, and the remaining parts of the left hemisphere were then treated overnight with 20% glycerol and 2% dimethylsulfoxide to prevent freeze-artifacts. The specimens were then embedded in a gelatin matrix using MultiBrain®Technology (NeuroScience Associates, Knoxville, TN). The blocks were rapidly frozen, after curing by immersion in 2-methylbutane chilled with crushed dry ice and mounted on a freezing stage of an AO 860 sliding microtome. The MultiBrain® blocks were sectioned coronally with 40 micrometers (μ) distance settings on the microtome. All sections were cut through the entire length of the specimen segment and collected sequentially into series of 24 containers. All containers contained Antigen Preserve solution (50% PBS pH7.0, 50% Ethylene Glycol, 1% Polyvinyl Pyrrolidone); no sections were discarded.

4.2.7. Delta FosB and OX-A immunohistochemistry

The delta FosB and OX-A immunohistochemistry were performed through the entire length of the brain hemisphere (minus olfactory bulbs). The specimens were sectioned at 40 μm and every 12th section, spaced at 480 μm intervals, was processed. The free-floating sections were stained with the desired stain. All incubation solutions from the primary antibody onward used Tris-buffered saline (TBS) with Triton X-100 as the vehicle; all rinses were with TBS. For delta FosB immunohistochemistry, after a hydrogen peroxide treatment, the sections were immunostained with a rabbit anti-delta FosB primary antibody (Catalog# 14695S, Cell Signaling, dilution 1:5000), overnight at room temperature. Vehicle solutions contained Triton X-100 for permeabilization. Following rinses, a biotinylated goat anti-rabbit IgG secondary antibody (Catalog#: BA-

1000, Vector, dilution 1:1000) was applied. After further rinses, Vector Lab's ABC solution (avidin-biotin-HRP complex; VECTASTAIN® Elite ABC, Vector, Burlingame, CA) was applied. The sections were again rinsed, then treated with diaminobenzidine tetrahydrochloride (DAB) with nickel and hydrogen peroxide to create a visible reaction product. Following further rinses, and a light counterstain with neutral red, the sections were mounted on gelatin-coated glass slides, and air dried. The slides were dehydrated in alcohol, cleared in xylene and coverslipped. For OX-A immunohistochemistry, after a hydrogen peroxide treatment, the sections were immunostained with a mouse anti-OX-A primary antibody (Catalog#: sc-80263, Santa Cruz, dilution 1:1500), overnight at room temperature. Vehicle solutions contained Triton X-100 for permeabilization. Following rinses, a biotinylated horse anti-mouse IgG secondary antibody (Catalog#: BA-2001, Vector, dilution 1:1000) was applied. After further rinses, Vector Lab's ABC solution (avidin-biotin-HRP complex; VECTASTAIN® Elite ABC, Vector, Burlingame, CA) was applied. The sections were again rinsed, then treated with DAB with nickel and hydrogen peroxide to create a visible reaction product. Following further rinses, and a light counterstain with neutral red, the sections were mounted on gelatin-coated glass slides, and air dried. The slides were dehydrated in alcohol, cleared in xylene and coverslipped.

4.2.8. Delta FosB/OX-A double immunofluorescent histochemistry

Immunofluorescent histochemistry was performed through the entire lateral hypothalamus (LH) (bregma from –1.3 to –5.2 mm), sectioned at 40 μ, to reveal delta FosB activity and OX-A positive neurons, counterstained with Hoechst, on every 6th section, spaced at 240 μ intervals. The free-floating sections were stained with the

desired stain. All incubation solutions from the primary antibody onward used TBS with Triton X-100 as the vehicle; all rinses were with TBS. The sections were immunostained with rabbit anti-delta FosB (Catalog# 14695S, Cell Signaling, dilution 1:150) and mouse anti-OX-A (Catalog#: sc-80263, Santa Cruz, dilution 1:150) primary antibodies, overnight at room temperature. Vehicle solutions contained Triton X-100 for permeabilization. Following rinses, Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Catalog#: 711-545-152, Jackson Lab, dilution 1:500) and Cy3-conjugated donkey anti-mouse IgG (Catalog#: 715-165-151, Jackson Lab, dilution 1:500) secondary antibodies were applied. Following rinses, Hoechst (a nissl counterstain) was applied. Following further rinses, the sections were mounted on gelatin-coated glass slides, and air dried. The slides were dehydrated in alcohol, cleared in xylene and coverslipped.

4.2.9. Quantification of delta FosB-positive cells and OX-A positive fibers

For delta FosB and OX-A immunohistochemistry, the slides were scanned using the Hamamatsu NanoZoomer S60 Digital slide scanner C13210-01 (resolution 20X) to obtain high-quality images of the sections for subsequent analysis. To analyze the number of delta FosB-positive cells and the area covered by OX-A immunoreactive fibers, the digital analysis was performed using the QuPath software (Bankhead et al., 2017). The algorithms for the detection of positive cells and fibers were based on a cell detection and a pixel classifier method, respectively, and software was trained on representative pictures with dedicated annotations. For delta FosB immunohistochemistry, the software was trained to recognize the darkly stained cells (delta FosB-positive cells) using the cell detection, and a threshold level for the optical

density (darkness) was selected to classify the delta FosB-positive cells as High-expressing (above) or Low-expressing (below), as illustrated in Figure 10. The number of positive cells was automatically counted in the brain region of interest, annotated according to the rat brain atlas (Paxinos & Watson, 2006) , and divided by the area of the region of interest drawn (Number of cells/mm^2). For delta FosB immunohistochemistry, the regions of interest were: the medial PFC (mPFC) [Prelimbic (PR) (+5.16 to +2.56 mm from Bregma) and Infralimbic (IL) (+3.72 to +2.56 mm from Bregma) cortices;]; the NAc Core (NAcC) and Shell (NAcSh) (+2.76 to +0.96 mm from Bregma); the VTA (–4.68 to –5.04 mm from Bregma); the Dorsal Striatum (caudate putamen; CPu) (+1.80 to –0.36 mm from Bregma) and the Paraventricular Hypothalamic Nucleus (PVN) (–1.08 to –1.92 mm from Bregma). The CPu was sub-divided into 4 regions, according to the previously described method (Sato et al., 2011). Briefly, the CPu ventral to the ventral limit of the lateral ventricle was classified as CPu Ventral, while the remaining CPu was divided into 3 zones of equal medial-lateral width, identified as CPu Lateral, CPu Intermediate and CPu Medial (see Figure 11). For OX-A immunohistochemistry, the QuPath software was trained to specifically recognize the OX-A positive fibers and to distinguish them from the background signals, using a pixel classifier (Figure 12 a). The area occupied by OX-A-positive fibers, expressed in mm^2, was automatically calculated in the annotated brain regions of interest, and then divided by the total area of the annotated region (mm^2/mm^2). The brain regions of interest were the mPFC [PR (+5.16 to +2.56 mm from Bregma) and IL (+3.72 to +2.56 mm from Bregma) cortices]; the VTA (–4.68 to –5.04 mm from Bregma); the Bed Nucleus of the Stria Terminalis (BNST) (+0.24 to –0.60 mm from Bregma), the CeA (–1.56 to –2.92 mm from Bregma), the PVT (–1.08 to –4.08 mm from Bregma),

the LC (–9.48 to –10.2 mm from Bregma) and the DRN (–6.96 to –9.24 mm from Bregma).

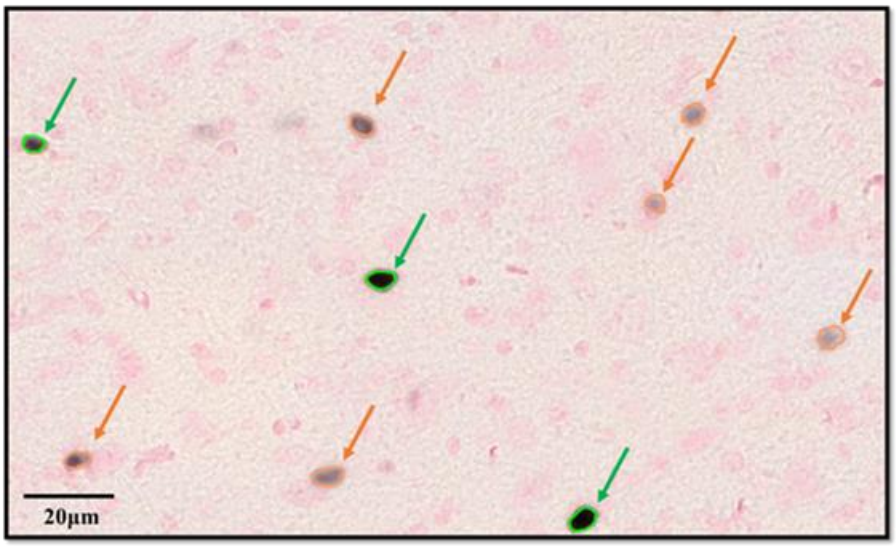


Figure 10. Representative picture illustrating the identification of delta FosB-positive cells and their distinction in High-expressing or Low-expressing cells. To recognize delta FosB-positive cells, the QuPath software (Bankhead et al., 2017) was trained to recognize all darkly stained cells (delta FosB-positive cells) using the cell detection method. After the detection, a threshold level for the optical density (darkness) was selected to classify the delta FosB-positive cells further as High-expressing (above) or Low-expressing (below). In the picture, the High-expressing cells are highlighted by the green arrows, while the Low-expressing cells are highlighted by the orange arrows.

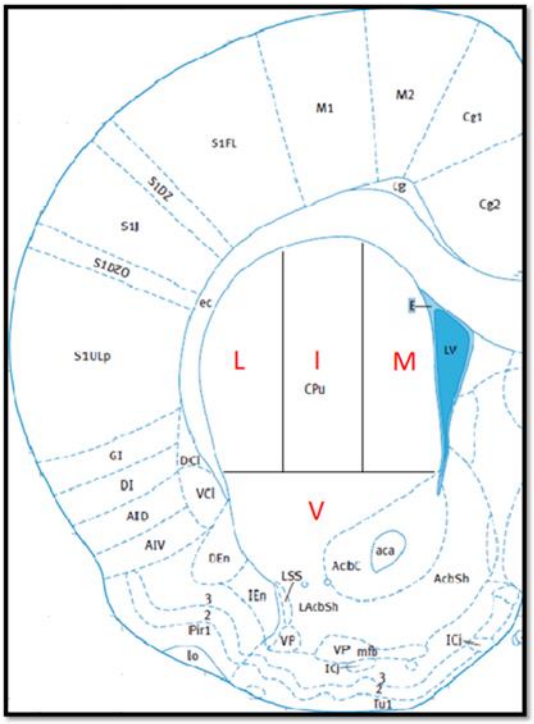


Figure 11. The partitioning scheme used to subdivide the Dorsal Striatum (caudate putamen; CPu) into the Medial (M), Intermediate (I), Lateral (L), and Ventral (V) zones for the quantification of delta FosB-positive cells. The CPu was sub-divided into 4 regions, according to the method used by (Sato et al., 2011). The CPu ventral to the ventral limit of the lateral ventricle was classified as CPu Ventral (V), while the remaining CPU was divided into 3 zones of equal medial-lateral width, identified as CPU Lateral (L), CPU Intermediate (I) and CPU Medial (M).

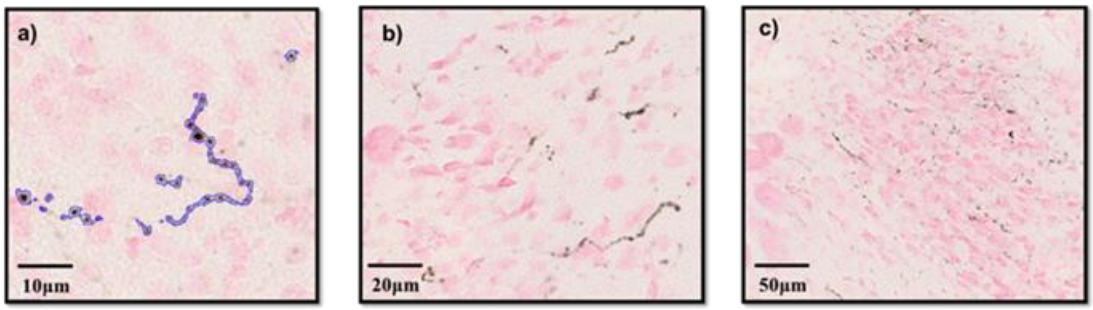


Figure 12. Representative pictures illustrating the identification of OX-A positive fibers and their distinction from background signals. The QuPath software was trained to specifically recognize the OX-A positive fibers (encompassed by the blue line in subpanel a) and to distinguish them from the

background signals, using a pixel classifier (a). The area occupied by the positive fibers was expressed in mm^2, and it was automatically calculated in the annotated brain regions of interest. (b, c): Representative pictures illustrating the OX-A positive fibers in the locus coeruleus at different magnifications. OX-A, orexin A.

4.2.10. Quantification of delta FosB and OX-A expressing neurons in the hypothalamus

The slides for delta FosB/OX-A double immunofluorescent histochemistry were scanned (20X resolution) at NeuroScience Associates (Knoxville, TN, USA) to obtain high-quality images for subsequent analysis. To analyze the number of delta FosB and OX-A-positive neurons in the hypothalamus, the digital analysis was performed using the QuPath software (Bankhead et al., 2017). To identify the number of delta FosB-positive cells (green), the software was trained to recognize the clearly distinguishable circular green cells using a pixel classifier, differentiating them from the background signals. After applying the pixel classifier, an optical density threshold was selected to classify the green cells as delta FosB-positive (above) or not (below) (Figure 13 b). To identify the number of OX-A-positive neurons (red), the software was trained to recognize the clearly distinguishable red cells and to differentiate them from the OX-A-positive fibers, using the cell detection (Figure 13 b). The colocalization of delta FosB- and OX-A-positive neurons was determined by initially identifying the OX-A-positive neurons, using the cell detection method, and then applying the pixel classifier for delta FosB, specifically on these neurons. The neurons that were positive for both the OX-A-cell detection and the delta FosB-pixel classifier were considered colocalized (Figure 13 b). The colocalization was expressed in terms of percentage of colocalized cells with respect to the total number of OX-A-positive neurons. The number of OX-A- and delta

FosB-positive cells, and the degree of their colocalization were calculated in three hypothalamic regions: the LH (from –1.72 to –4.44 mm from the Bregma), the DMH (from –2.40 to –3.60 mm from the Bregma) and the PeF (from –2.40 to –3.84 mm from the Bregma), annotated according to the rat brain atlas (Paxinos & Watson, 2006) (Figure 13 a).

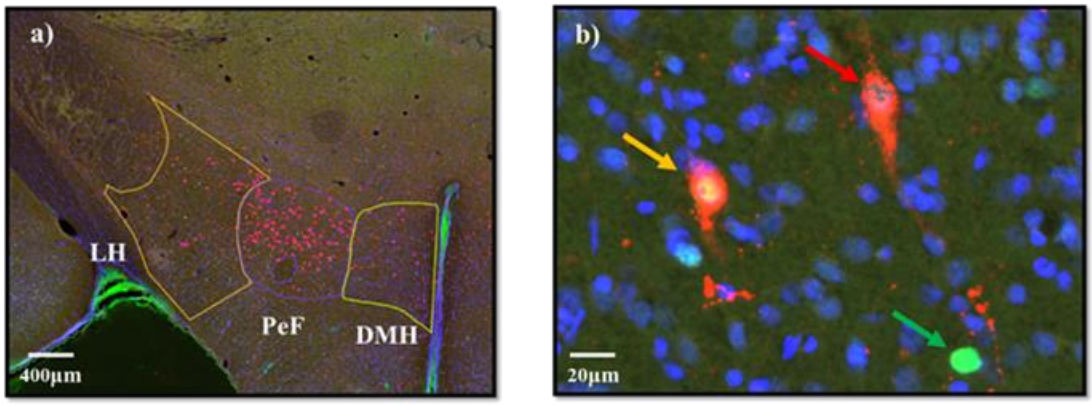


Figure 13. Representative pictures illustrating the subdivision of the hypothalamus in the LH, PeF and DMH, and the identification of delta FosB and OX-A positive neurons using immunofluorescent staining. (a) Representative picture showing the subdivision of the hypothalamus in the LH, PeF and DMH, annotated according to the rat brain atlas. (b) The picture shows examples of delta FosB-positive cells (green) and OX-A positive neurons (red) in the hypothalamus. The QuPath software was trained to identify the number of clearly distinguishable circular green cells (green arrow) using a pixel classifier. After applying the pixel classifier, an optical density threshold was selected to classify the green cells as delta FosB-positive (above) or not (below). To identify the number of OX-A positive neurons, the software was trained to recognize the clearly distinguishable red cells (red arrow) and to differentiate them from the OX-A positive fibers, using the cell detection module. The neurons that co-expressed both delta-FosB and OX-A (yellow cells; yellow arrow) were quantified by initially identifying the OX-A positive cells, using the cell detection method, and then applying on them the pixel classifier for delta FosB. The neurons that were positive for both the OX-A-cell detection module and the delta FosB-pixel classifier module were considered colocalized. DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; OX-A, orexin A; PeF, perifornical area.

4.2.11. Statistical analysis

Body weights (g) and HPF intake (kcal/kg) data were expressed as mean ± SEM. The two-tailed unpaired t-test was used to compare the amount of HPF ingested by vehicle-treated NR + NS rats and R + S rats, and to compare the HPF intake of vehicle- and ACT-539313-treated NR + NS and R + S rats on experimental days (day 25 and 35). The two-way ANOVA test, followed by the Tukey's post-hoc test to correct for multiple comparisons, was used to compare the number of delta FosB- and OX-A-positive cells, and the amount of OX-A-positive fibers in vehicle- or ACT-539313-treated R + S and NR + NS rats. The statistical analysis included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs 15 mg/kg ACT-539313). The two-tailed unpaired t-test was used to compare the amount of OX-A-positive fibers in the CeA, DRN and PVT in NR + NS and R + S rats, independently from the treatment (Vehicle vs ACT-539313), and to compare the amount of OX-positive neurons and delta FosB-positive cells in the LH, PeF and DMH of rats treated with vehicle or ACT-539313 (independently from exposure (or not) to the binge eating protocol). Data are expressed as mean ± SEM. The statistical tests were performed using GraphPad version 8.1.1 (GraphPad Software Inc., La Jolla CA, USA). Differences were considered significant when $p < 0.05$.

4.3. Results

4.3.1. Effect of acute and chronic administration of ACT-539313 (15 mg/kg) on the binge eating episode

Figure 14 represents body weight fluctuations of rats during the four 8-day cycles in which we either exposed (R + S) or did not expose (NR + NS) the rats to food restriction

on days 1–4 of each cycle (66% of regular chow availability). In accordance with our previous studies (Cifani et al., 2009; Micioni Di Bonaventura et al., 2014; Micioni Di Bonaventura, Lutz, et al., 2017; Romano et al., 2020), we found that the rats lost weight during the 4-day food restriction periods and regained it during the subsequent 4-day free-food periods. On the test days (day 25 and 35) the body weight of the rats in the restricted and non-restricted groups was not significantly different.

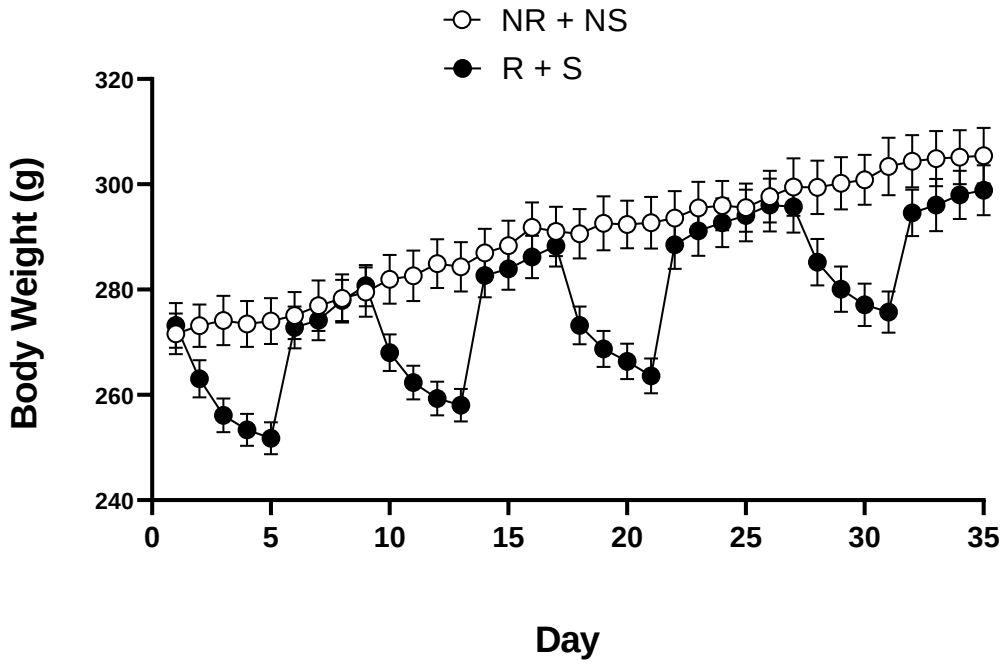


Figure 14. Mean ± SEM body weight (g) of female rats exposed or not exposed to repeated intermittent cycles of food restriction/refeeding. Data are expressed as mean ± SEM. NR + NS, non-restricted plus non-stressed rats; R + S, restricted plus stressed rats; SEM, standard error of the mean.

In accordance with our previous studies in the same animal model of binge eating (Cifani et al., 2009; Micioni Di Bonaventura et al., 2014; Micioni Di Bonaventura, Lutz, et al., 2017; Micioni Di Bonaventura, Ubaldi, et al., 2017; Romano et al., 2020), the two-tailed unpaired t-test revealed that on experiment days (day 25 and 35) vehicle-treated R + S rats significantly increased the HPF consumption compared to the vehicle-treated control

group, during the first 15 min (first time point evaluated) and the entire 120 min of the test (Day 25: 15 min time point, $p < 0,0001$; 120 min time point, $p < 0,0001$, Figure 15 A; Day 35: 15 min time point, $p = 0,0002$; 120 min time point, $p = 0,0002$, Figure 15 B).

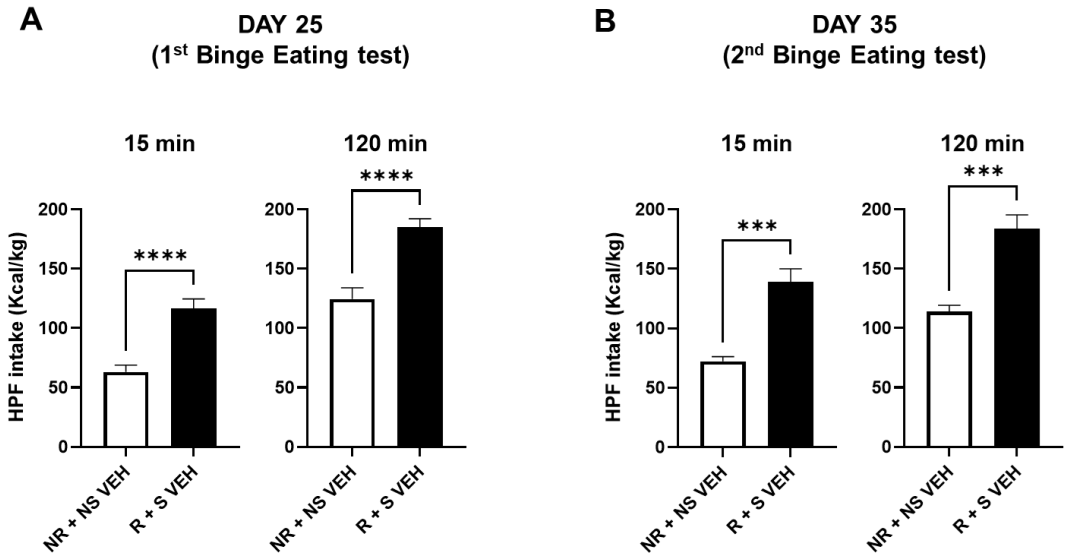


Figure 15. Establishment of binge-like eating behavior in female rats exposed or not exposed to repeated intermittent cycles of food restriction/refeeding plus frustration stress. (A) HPF intake of vehicle-treated NR + NS and R + S rats on day 25, expressed in kcal/kg, during the first 15 min (first time point evaluated) and the entire 120 min of the test. (B) HPF intake of vehicle-treated NR + NS and R + S rats on day 35, expressed in kcal/kg, during the first 15 min (first time point evaluated) and the entire 120 min of the test. Data are expressed as mean ± SEM. HPF, highly palatable food; NR + NS, non-restricted plus non-stressed rats; R + S, restricted plus stressed rats; SEM, standard error of the mean; VEH, vehicle-treated.

On day 25, the two-tailed unpaired t-test showed that acute pre-treatment with ACT-539313 was able to block the binge eating episode selectively in R + S rats, without influencing the HPF intake in NR + NS rats (Figure 16 A). The significant effect of compound administration in decreasing the HPF consumption in R + S rats was observed

during the first 15 min (first time point evaluated) and the entire 120 min of the test (Day 25: 15 min time point, p = 0,0028; 120 min time point, p < 0,0001; Figure 16 B). Similarly, chronic administration of the compound was effective in blocking the binge eating episode in R + S rats on day 35 (2nd binge eating test), showing again no signifiant effect on HPF consumption in NR + NS rats (Figure 16 C). The unpaired t-test revealed that the effect of ACT-539313 on HPF intake reduction in R + S rats was significant during the first 15 min (first time point evaluated) and the entire 120 min of the test (Day 35: 15 min time point, p = 0,0005; 120 min time point, p < 0,0012; Figure 16 D).

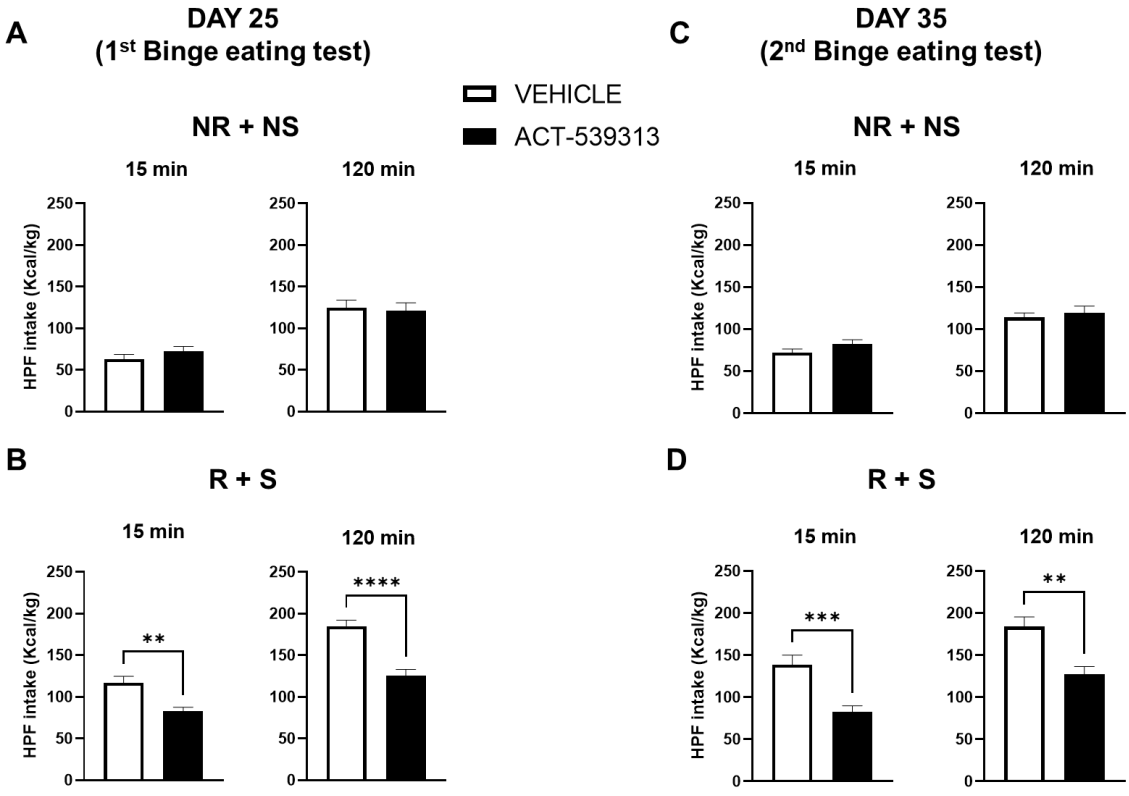


Figure 16. Effect of acute and chronic administration of ACT-539313 on the binge eating episode on day 25 and 35. (A) HPF intake, expressed in kcal/kg, of NR + NS rats treated with vehicle or ACT-539313 during the first 15 min (first time point evaluated) and the entire 120 min of the test, on day 25. (B) HPF intake, expressed in kcal/kg, of R + S rats treated with vehicle or ACT-539313 during the first 15 min (first time point evaluated) and the entire 120 min of the test, on day 25. (C) HPF intake, expressed

in kcal/kg, of NR + NS rats treated with vehicle or ACT-539313 during the first 15 min (first time point evaluated) and the entire 120 min of the test, on day 35. (D) HPF intake, expressed in kcal/kg, of R + S rats treated with vehicle or ACT-539313 during the first 15 min (first time point evaluated) and the entire 120 min of the test, on day 35. Data are expressed as mean ± SEM. HPF, highly palatable food; NR + NS, non-restricted plus non-stressed rats; R + S, restricted plus stressed rats; SEM, standard error of the mean.

4.3.2. Effect of exposure to the binge eating protocol and chronic treatment with ACT-539313 on the quantity of OX-A-positive fibers in extra-hypothalamic brain regions

In this experiment, I evaluated the effect of exposure to an additional cycle of restriction/re-feeding plus frustration stress and of chronic treatment with ACT-539313 on the amount of OXA-positive fibers in extra-hypothalamic brain regions implicated in binge eating behavior. The two-way ANOVA revealed no statistically significant effect of either "Treatment" (ACT-539313 vs Vehicle) or "Condition" (NR + NS vs R + S) on the average amount of OX-A-positive fibers in the mPFC (PR and IL cortices) (Figure 17 A, B), BNST, VTA and LC (Figure 18 A, B, C).

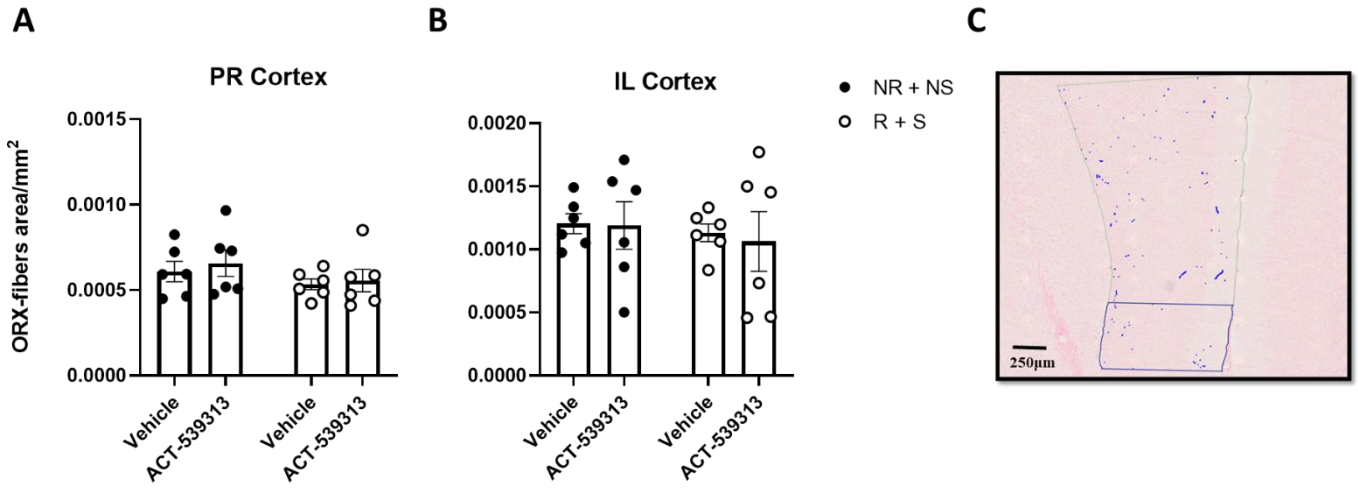


Figure 17. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the quantity of OX-A-positive fibers in the mPFC (PR and IF cortices). The graphs show the amount of OX-positive fibers in NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg), in the PR Cortex (A) and IL Cortex (B). The quantity of OX fibers was expressed in terms of area covered by the black fibers (mm^2) and divided by the area of the region of interest annotated (mm^2/mm^2). (C) Representative picture showing the subdivision of the mPFC in the PR and IF cortices and the identification of OX-positive fibers, encompassed by the blue lines. The ordinary two-way ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons was used to compare the quantity of OX-positive fibers in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. IL, infralimbic; NR + NS, non-restricted plus non-stressed ORX, orexin; PR, prelimbic; R + S, restricted plus stressed. SEM, standard error of the mean.

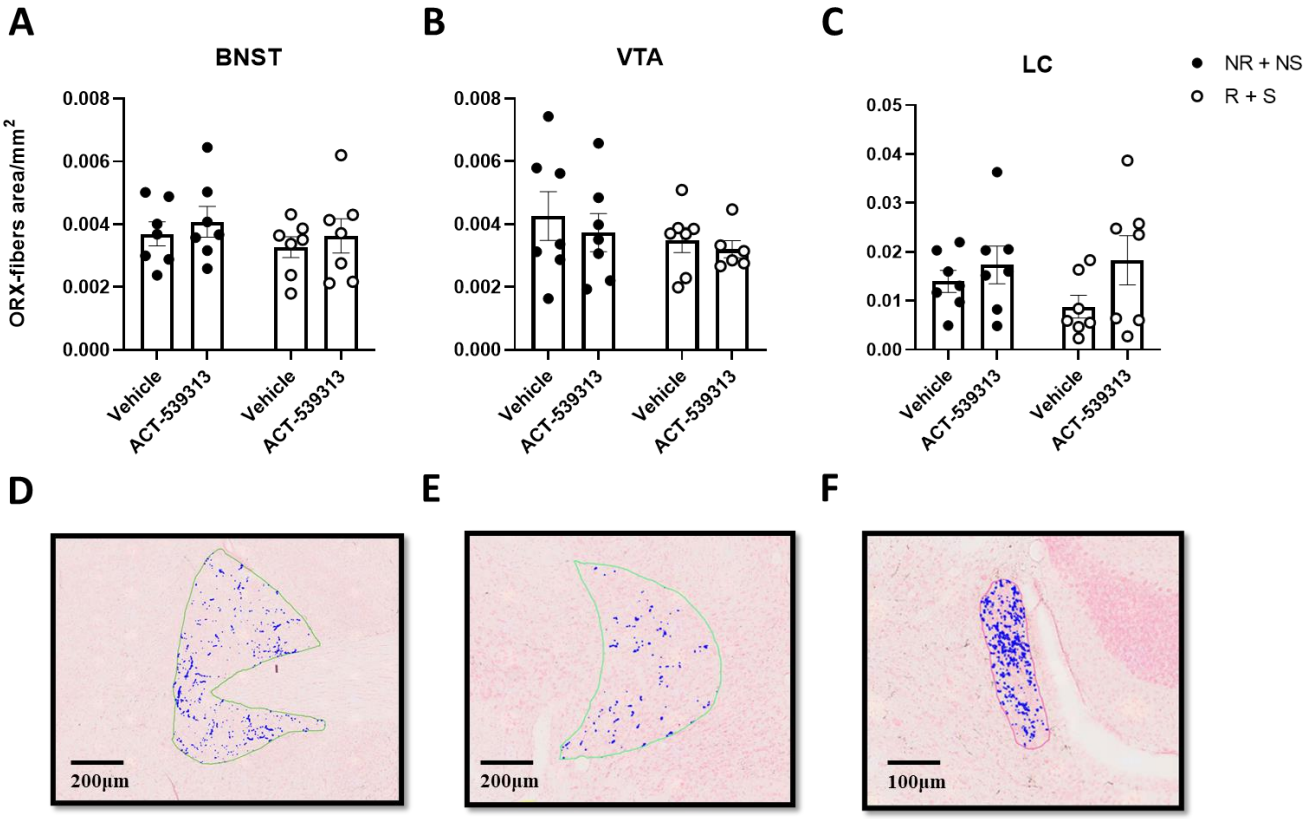


Figure 18. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the quantity of OX-A-positive fibers in the BNST, VTA and LC. The graphs show the amount of OX-positive fibers in NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg), in the BNST (A), VTA (B) and LC (C). The quantity of OX-positive fibers was expressed in terms of area covered by the black fibers (mm^2) and divided by the area of the region of interest annotated (mm^2/ mm^2). (D, E, F) Representative pictures showing the annotation of the BNST (D), VTA (E) and LC (F), and the identification of OX-positive fibers, encompassed by the blue lines. The ordinary two-way ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons was used to compare the quantity of OX-positive fibers in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. BNST, bed nucleus of the stria terminalis; LC, locus coeruleus; NR + NS, non-restricted + non-stressed; ORX, orexin; R + S, restricted plus stressed; SEM, standard error of the mean; VTA, ventral tegmental area.

Conversely, a statistically significant effect of "Condition" was found in both the CeA ($F_{(1, 24)} = 4.924$ $p = 0.0362$) (Figure 19 A) and DRN ($F_{(1, 22)} = 4.823$ $p = 0.0389$) (Figure 19 B), and a trend for significance was observed in the PVT ($F_{(1, 23)} = 4.254$ $p = 0.0506$) (Figure 19 C). In these three brain regions, the R + S vehicle and ACT-539313 treated rats appeared to display a reduced amount of OX-positive fibers compared to the NR + NS groups. To better analyze the different amount of OX-positive fibers in NR + NS and R + S rats in these brain regions, the unpaired t-test was performed in order to compare the amount of OX-fibers in these rat groups, independently from the treatment (Vehicle vs ACT-539313). The two-tailed, unpaired t-test revealed a significantly decreased amount of OX-positive fibers in the CeA ($p = 0,0326$) (Figure 20 A), DRN ($p = 0,0349$) (Figure 20 B) and PVT ($p = 0,0469$) (Figure 20 C) of R + S rats compared to the NR + NS group.

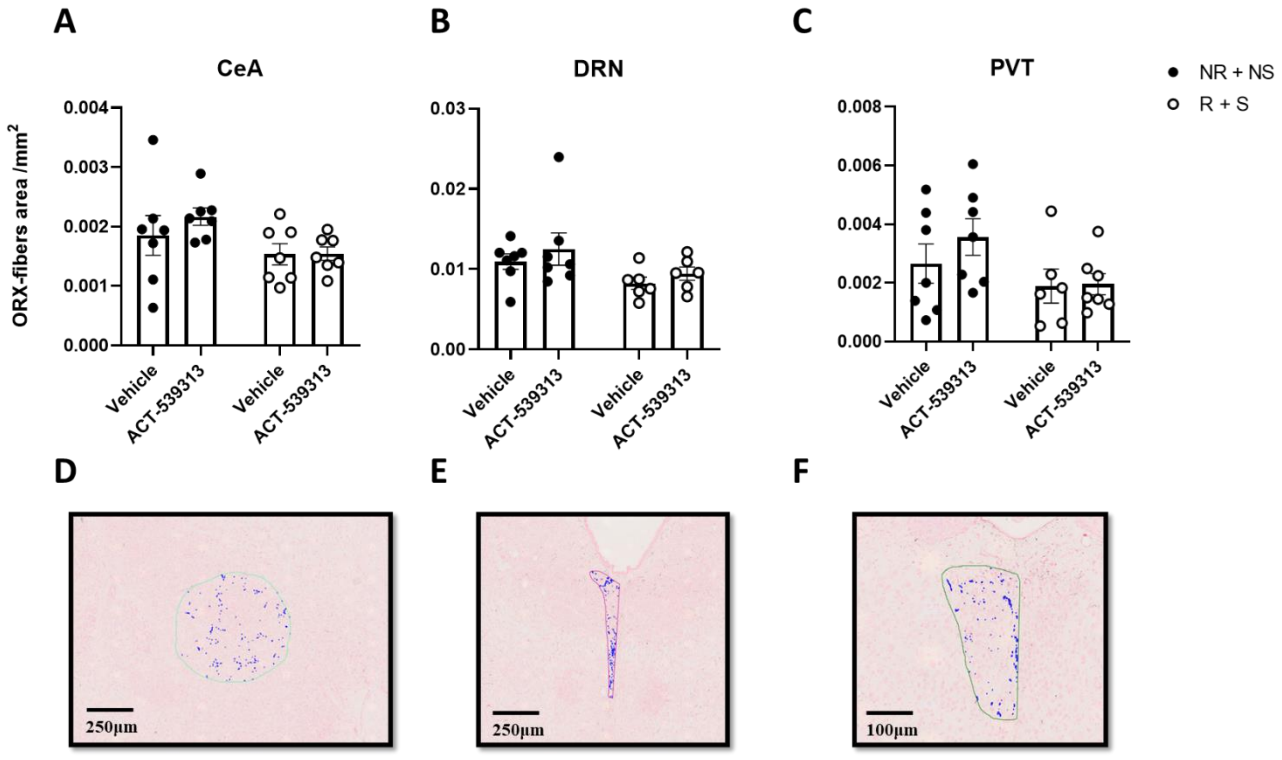


Figure 19. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the quantity of OX-A-positive fibers in the CeA, DRN and PVT. The graphs show the amount of OX-positive fibers in NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg), in the CeA (A), DRN (B) and PVT (C). The quantity of OX-fibers was expressed in terms of area covered by the black fibers (mm^2) and divided by the area of the region of interest annotated (mm^2/ mm^2). (D, E, F) Representative pictures showing the annotation of the CeA (D), DRN (E) and PVT (F), and the identification of OX-positive fibers, encompassed by the blue lines. The ordinary two-way ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons was used to compare the quantity of OX-positive fibers in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. CeA, central amygdala; DRN, dorsal raphe nucleus; NR + NS, non-restricted plus non-stressed; ORX, orexin; PVT, paraventricular thalamic nucleus; R + S, restricted plus stressed; SEM, standard error of the mean.

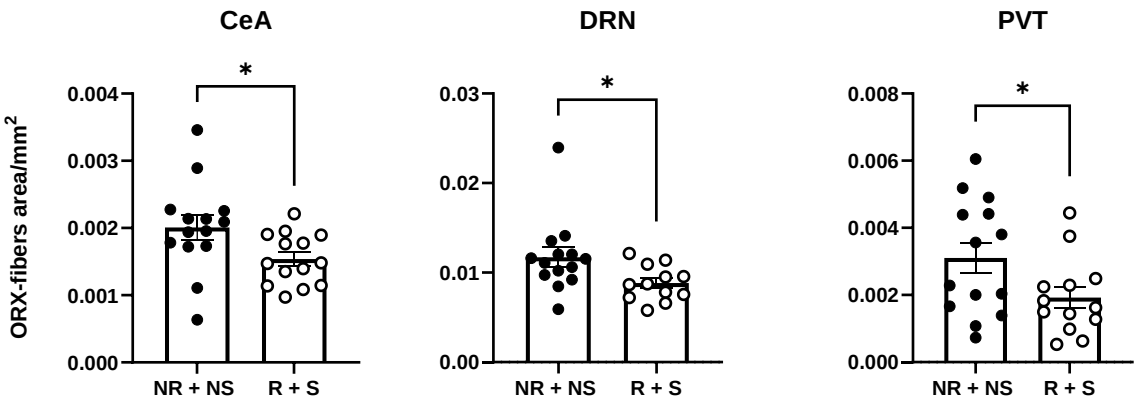


Figure 20. Effect of chronic exposure to the binge eating protocol on the quantity of OX-A-positive fibers in the CeA, DRN and PVT. The graphs show the amount of OX-positive fibers in NR + NS and R + S rats chronically exposed to the binge eating protocol in the CeA (A), DRN (B) and PVT (C). The quantity of OX-fibers was expressed in terms of area covered by the black fibers (mm^2) and divided by the area of the region of interest annotated (mm^2/ mm^2). The two-tailed, unpaired t-test was used to compare the quantity of OX-positive fibers in R + S and NR + NS rats, independently from the treatment. Data are expressed as mean ± SEM. CeA, central amygdala; DRN, dorsal raphe nucleus; NR + NS, non-restricted plus non-stressed; ORX, orexin; PVT, paraventricular thalamic nucleus; R + S, restricted plus stressed; SEM, standard error of the mean.

4.3.3. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the transcription factor delta FosB in multiple brain regions

In this experiment, the two-way ANOVA did not reveal a statistically significant effect of either "Treatment" or "Condition" on the total number of delta FosB-positive cells, or on High-expressing or Low-expressing cells, in any of the brain regions investigated: the mPFC (PR and IL cortices) (Figure 21 A, B, C, D, E, F), NAcC and NacSh (Figure 22 A, B, C, D, E, F), VTA (Figure 23 A, B, C), PVN (Figure 24 A, B, C), the Ventral

CPu (Figure 25 A, B, C), Lateral CPu (Figure 25 D, E, F), Intermediate CPu (Figure 25 G, H, I), Medial (Figure 25 J, K, L) or Total CPu (Figure 25 M, N, O).

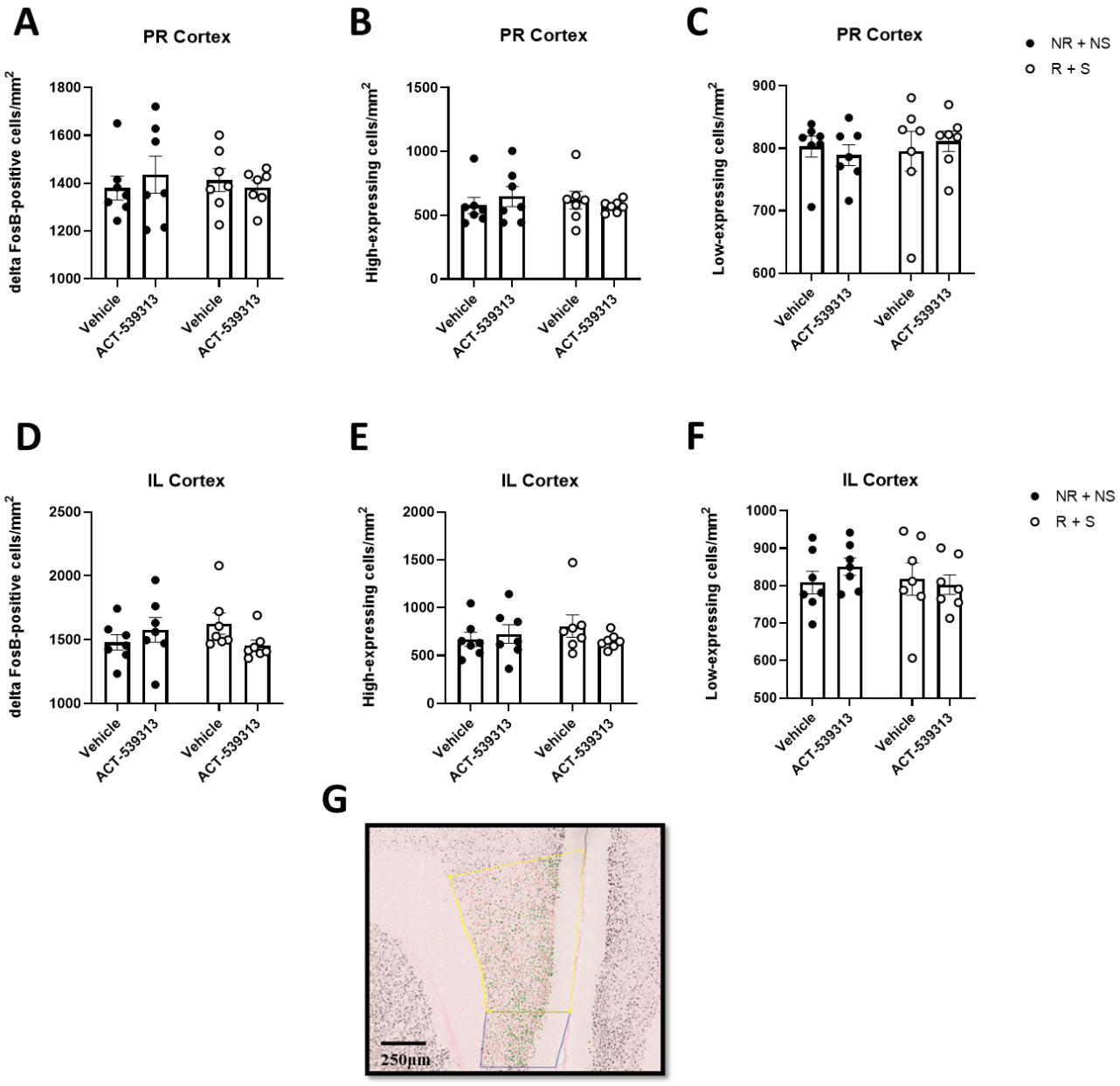


Figure 21. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the transcription factor delta FosB in the mPFC (PR and IL cortices). The graphs show the total number of delta FosB-positive cells, High-expressing cells and Low-expressing cells, in NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg), in the PR Cortex (A, B, C) and IL Cortex (D, E, F). The number of all types of delta FosB-positive cells was divided by

the area of the region of interest annotated (Number of cells/ mm²). (G) Representative picture showing the subdivision of the mPFC in the PR and IF cortices and the detection of delta FosB-positive cells. The ordinary two-way ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons, was used to compare the number of delta FosB-positive cells in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. IL, infralimbic; NR + NS, non-restricted plus non-stressed; PR, prelimbic; R + S, restricted plus stressed, SEM, standard error of the mean.

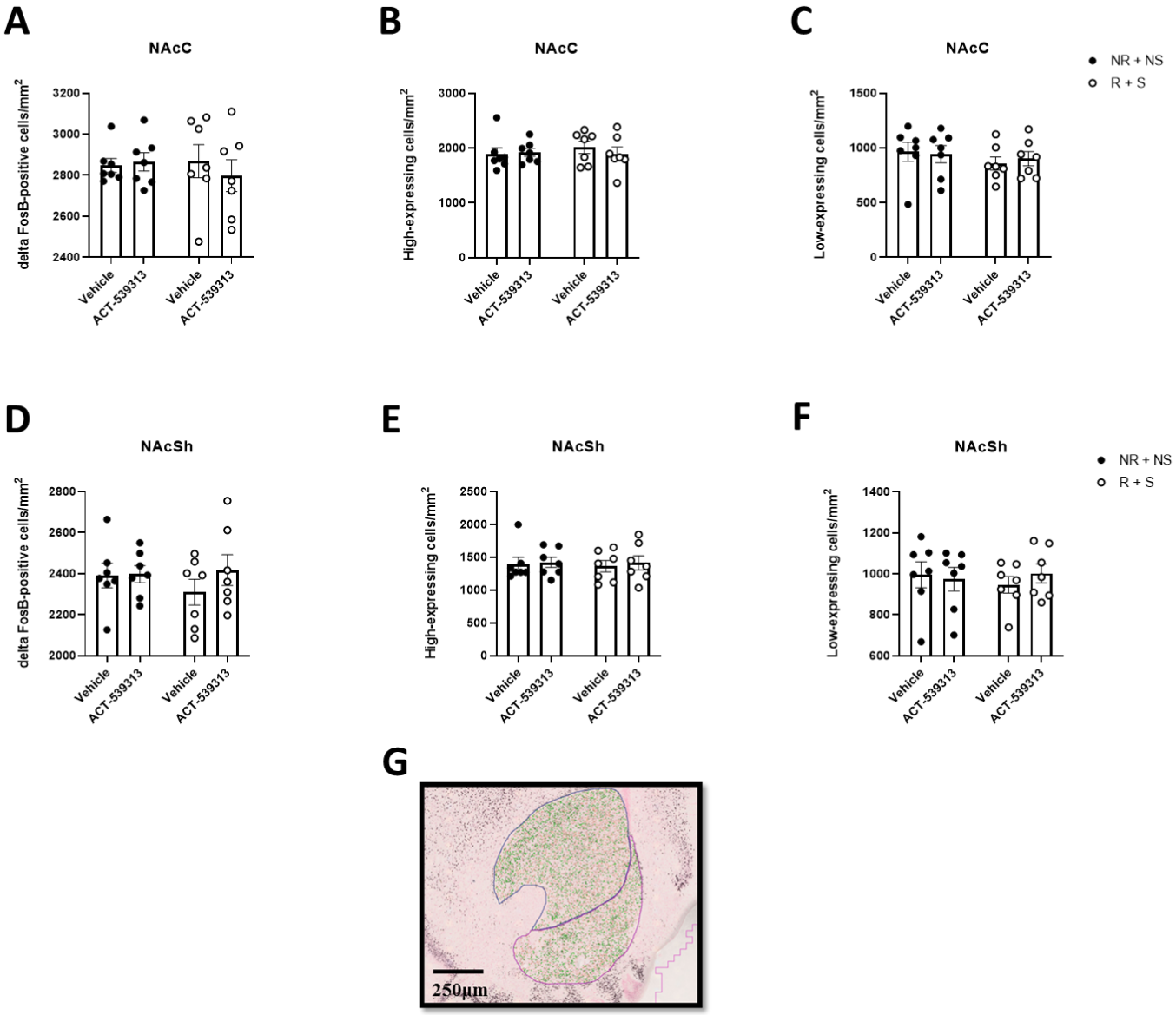


Figure 22. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the transcription factor delta FosB in the NacC and NacSh. The graphs show the total number of delta FosB-positive cells, High-expressing cells and Low-expressing cells, in NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg), in the NacC (A, B, C) and NacSh (D, E, F). The number of all types of delta FosB-positive cells was divided by the area of the region of interest annotated (Number of cells/ mm²). (G) Representative picture showing the subdivision of the NAc in the NacC and NacSh, and the detection of delta FosB-positive cells. The ordinary two-way ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons was used to compare the number of delta FosB-positive cells in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. NacC, nucleus accumbens core; NacSh, nucleus accumbens shell; NR + NS, non-restricted plus non-stressed; R + S, restricted plus stressed; SEM, standard error of the mean.

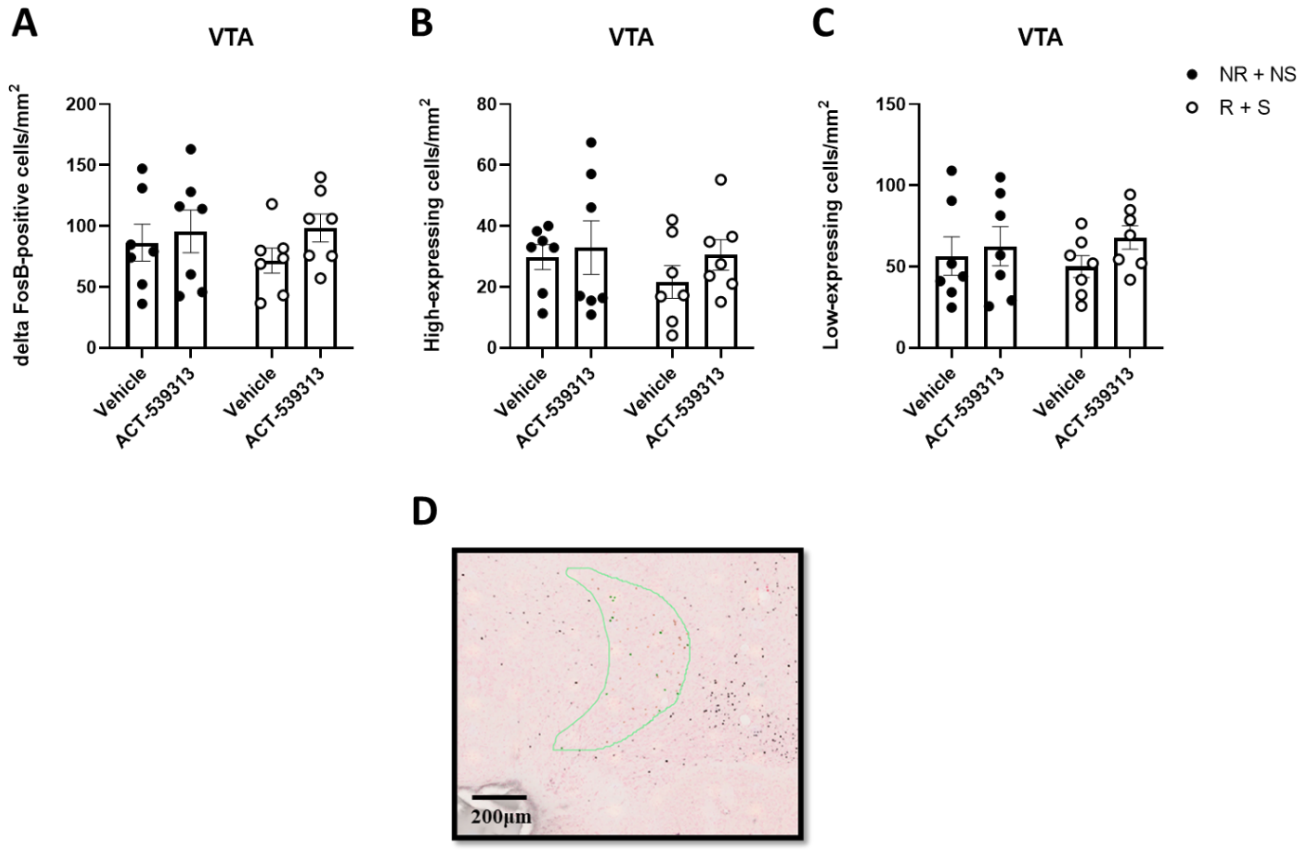


Figure 23. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the transcription factor delta FosB in the VTA. The graphs show the total number of delta FosB-positive cells, High-expressing cells and Low-expressing cells, in NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg), in the VTA (A, B, C). The number of all types of delta FosB-positive cells was divided by the area of the region of interest annotated (Number of cells/mm²). (D) Representative picture showing the VTA and the detection of delta FosB-positive cells. The ordinary two-way ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons was used to compare the number of delta FosB-positive cells in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. NR + NS non-restricted plus non-stressed; R + S, restricted plus stressed; SEM, standard error of the mean; VTA, ventral tegmental area.

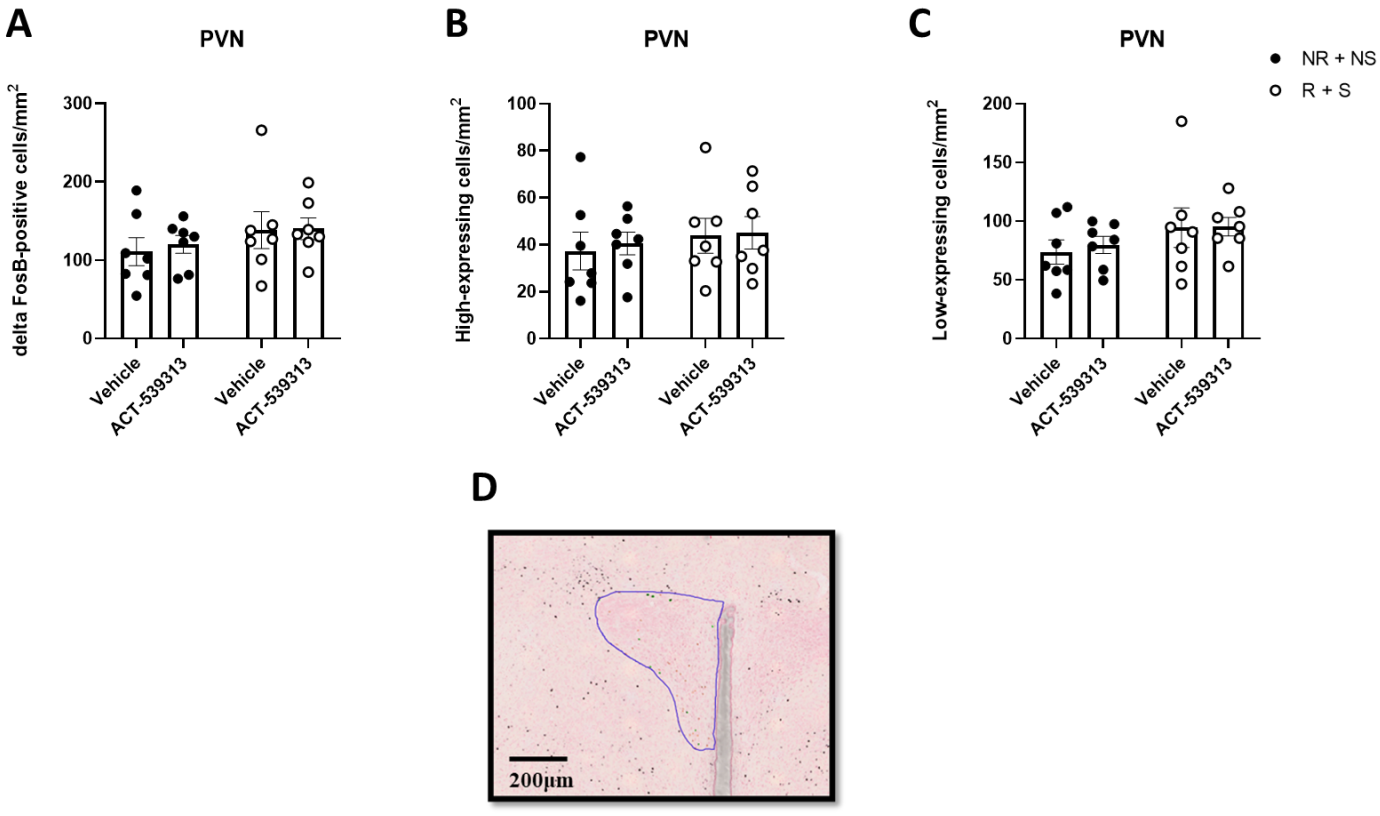


Figure 24. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the transcription factor delta FosB in the PVN. The graphs show the total number of delta FosB-positive cells, High-expressing cells and Low-expressing cells, in NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg), in the PVN (A, B, C). The number of

all types of delta FosB-positive cells was divided by the area of the region of interest annotated (Number of cells/ mm^2). (d) Representative picture showing the PVN and the detection of delta FosB-positive cells. The ordinary two-way ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons was used to compare the number of delta FosB-positive cells in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. NR + NS, non-restricted plus non-stressed; PVN, paraventricular hypothalamic nucleus; R + S, restricted plus stressed; SEM, standard error of the mean.

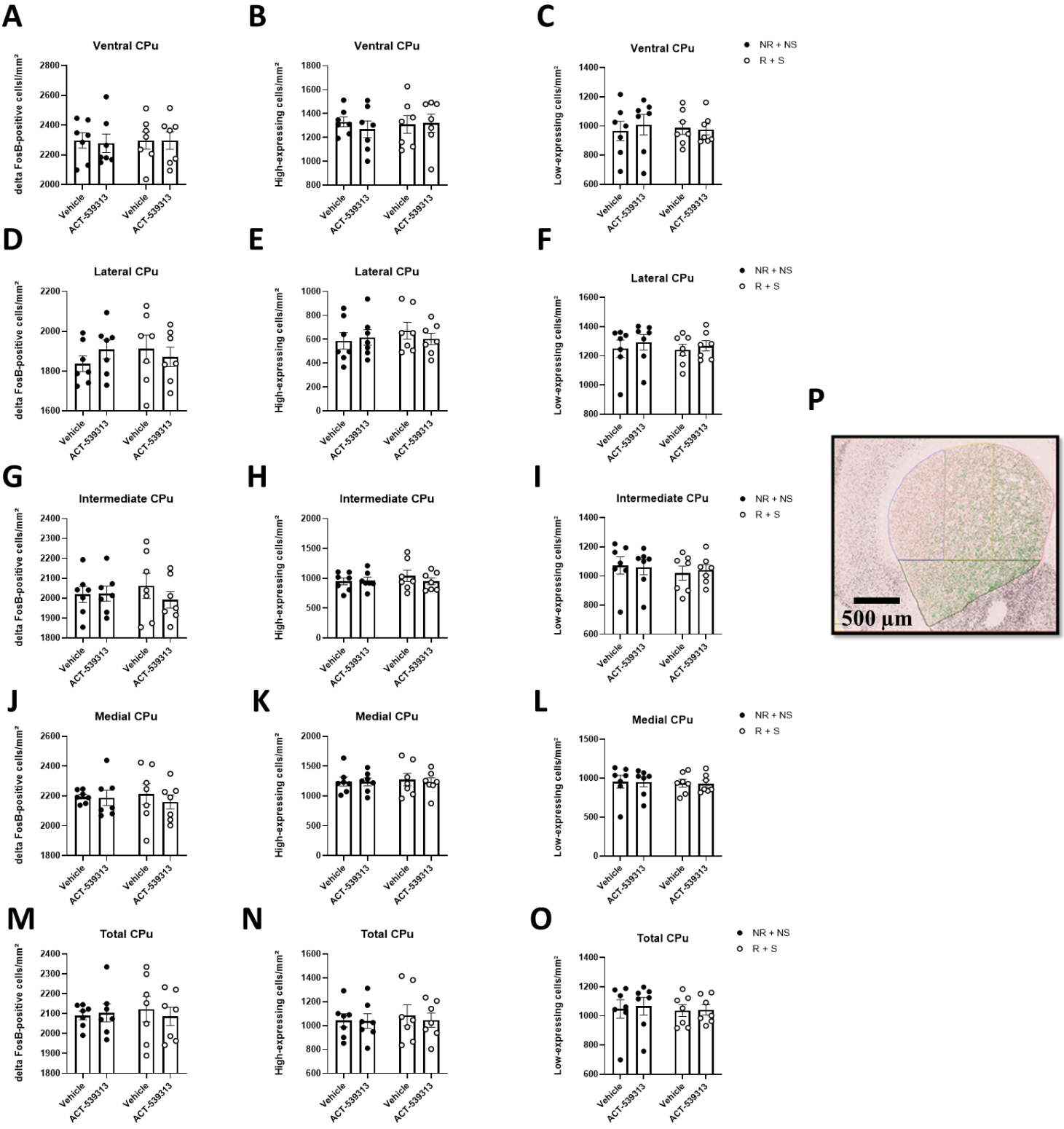


Figure 25. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the transcription factor delta FosB in the CPu (Ventral, Lateral, Intermediate, Medial part, and Total CPu). The graphs show the total number of delta FosB-positive cells, High-

expressing cells and Low-expressing cells, in NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg), in the Ventral (A, B, C), Lateral (D, E, F), Intermediate (G, H, I), Medial (J, K, L) and Total Cpu (M, N, O). The number of all types of delta FosB-positive cells was divided by the area of the region of interest annotated (Number of cells/mm²). (P) Representative pictures showing the division of the CPu in the four subregions (Ventral, Lateral, Intermediate, Medial) and the detection of Delta FosB-positive cells. The ordinary two-way ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons was used to compare the number of delta FosB-positive cells in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. CPu, caudate putamen; NR + NS, non-restricted; plus non-stressed; R + S, restricted plus stressed; SEM, standard error of the mean.

4.3.4. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on hypothalamic OX-expressing neurons and delta FosB-positive cells

In the last experiment, the two-way ANOVA revealed a statistically significant effect of "Treatment" on the total quantity of OX-neurons in the LH ($F_{(1, 24)} = 8.647$ $p = 0.0084$) (Figure 26 A) and in the PeF ($F_{(1, 24)} = 2.899$ $p = 0.0340$) (Figure 26 B), and a trend for significance in the DMH ($F_{(1, 24)} = 3.574$ $p = 0.0828$) (Figure 26 C). The rats treated with the OX1R antagonist appeared to show a higher quantity of OX-expressing neurons, in all the three hypothalamic regions. To better analyze the different amount of OX-neurons in vehicle- or ACT-539313-treated rats in these hypothalamic subregions, the unpaired t-test was performed in order to compare the amount of OX-neurons in these rat groups, independently from the exposure (or not) to the binge eating protocol. The two-tailed, unpaired t-test revealed a significantly increased amount of OX-neurons in the LH ($p = 0,0063$) (Figure 27 A) and PeF ($p =$

0,0282) (Figure 27 B), and a trend for significance in the DMH (p = 0,0775) (Figure 27 C).

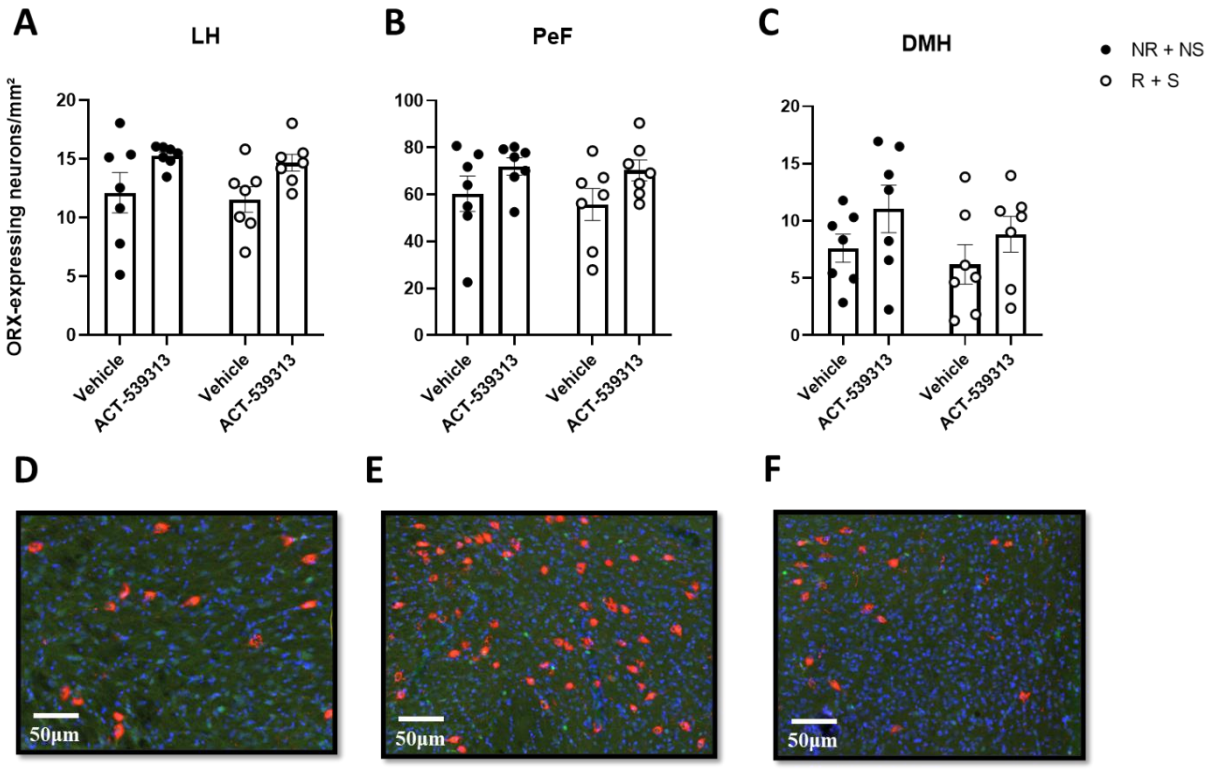


Figure 26. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on OX-expressing neurons in the LH, PeF and DMH. The graphs show the total number of OX-positive neurons in the LH (A), PeF (B) and DMH (C) of NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg). The OX-positive neurons were identified using a cell detection method, and the number of neurons was divided by the area of the region of interest annotated (Number of cells/mm2). (D, E, F) Representative pictures showing the identification of OX-positive neurons in the LH, PeF and DMH. The ordinary two-way ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons was used to compare the quantity of OX-positive neurons in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. DMH, dorsomedial hypothalamus; LH, lateral hypothalamus;

NR + NS, non-restricted plus non-stressed; ORX, orexin; PeF, perifornical area; R + S, restricted plus stressed; SEM, standard error of the mean.

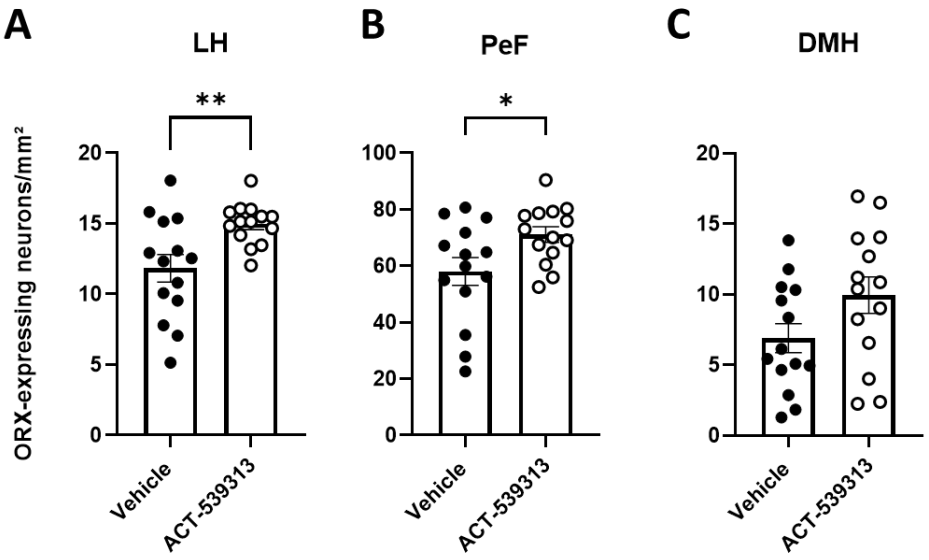


Figure 27. Effect of chronic treatment with ACT-539313 on OX-expressing neurons in the LH, PeF and DMH. The graphs show the total number of OX-positive neurons in the LH (A), PeF (B) and DMH (C) of rats chronically treated with vehicle or ACT-539313 (15 mg/kg). The OX-positive neurons were identified using a cell detection method, and the number of neurons was divided by the area of the region of interest annotated (Number of cells/mm2). The two-tailed, unpaired t-test was used to compare the quantity of OX-positive neurons in vehicle- or ACT-539313-treated rats, independently of the exposure (or not) to the binge eating protocol. Data are expressed as mean ± SEM. DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; ORX, orexin; PeF, perifornical area; SEM, standard error of the mean

Regarding hypothalamic delta FosB-positive cells, the two-way ANOVA revealed only a statistically significant effect of "Treatment" in the PeF ($F_{(1, 24)} = 5.439$ $p = 0.0284$) (Figure 28 B) and a trend for significance in the LH ($F(1, 24) = 3.070$ $p = 0.0925$) (Figure 28 A). The rats treated with the OX1R antagonist appeared to show a

higher amount of delta FosB-positive cells in these hypothalamic sub-regions. To better analyze the different amount of delta FosB-positive cells in vehicle- or ACT-539313-treated rats in these hypothalamic subregions, the unpaired t-test was performed in order to compare the amount of delta FosB-positive cells in these rat groups, independently from the exposure (or not) to the binge eating protocol. The two-tailed, unpaired t-test revealed a significantly increased amount of delta FosB-positive cells in the PeF (p = 0,0283) (Figure 29 B) and a trend for significance in the LH (p = 0,0950) (Figure 29 A).

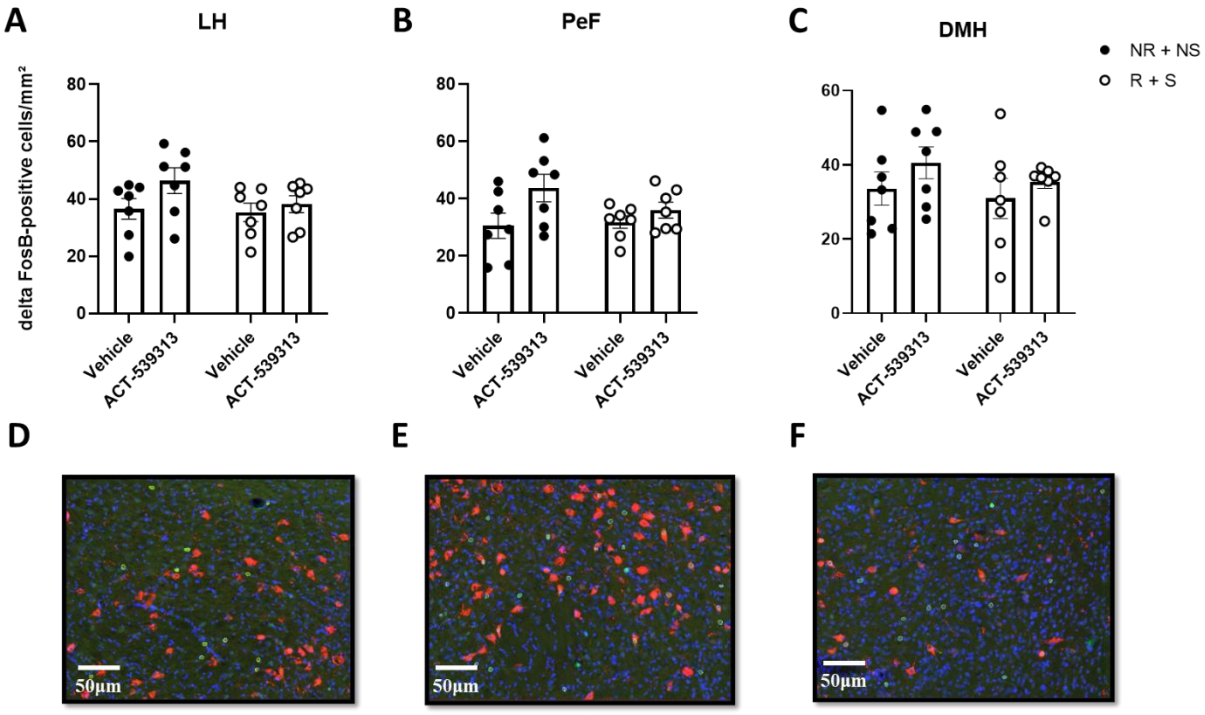


Figure 28. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the transcription factor delta FosB in the LH, PeF and DMH. The graphs show the total number of delta FosB-positive cells in the LH (A), PeF (B) and DMH (C) of NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg). The delta FosB-positive cells were identified using a pixel classifier method, and the number of identified cells was divided by the area of the region of interest annotated (delta FosB-positive cells/mm^2). (D, E, F) Representative pictures showing the identification of delta FosB-positive cells in the LH, PeF and DMH. The ordinary two-way

ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons was used to compare the number of delta FosB-positive cells in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; NR + NS, non-restricted plus non-stressed; PeF, perifornical area; R + S, restricted plus stressed; SEM, standard error of the mean.

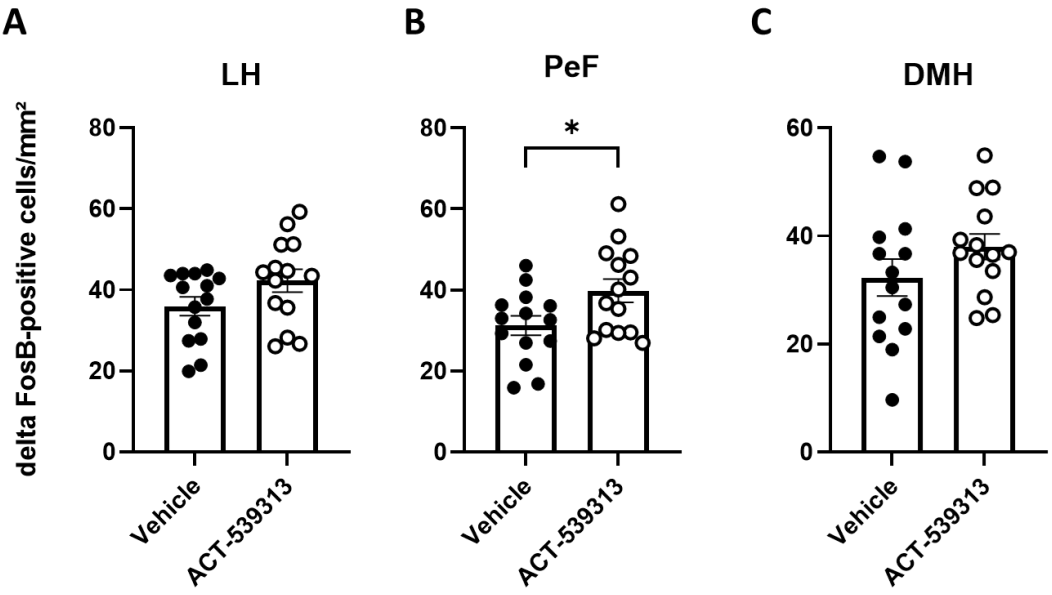


Figure 29. Effect of chronic treatment with ACT-539313 on the transcription factor delta FosB in the LH, PeF and DMH. The graphs show the total number of delta FosB-positive cells in the LH (A), PeF (B) and DMH (C) of rats chronically treated with vehicle or ACT-539313 (15 mg/kg), independently of exposure (or not) to the binge eating protocol. The delta FosB-positive cells were identified using a pixel classifier method, and the number of identified cells was divided by the area of the region of interest annotated (delta FosB-positive cells/mm^2). The two-tailed, unpaired t-test was used to compare the number of delta FosB-positive cells in vehicle- or ACT-539313-treated rats. Data are expressed as mean ± SEM. DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; PeF, perifornical area; SEM, standard error of the mean.

Finally, we analyzed the percentage of colocalization of delta FosB within OX-expressing neurons. Generally, the degree of colocalization was relatively low in the all three hypothalamic sub-regions. The two-way ANOVA revealed only a statistically significant effect of "Condition" ($F_{(1, 24)} = 6.278$ $p = 0.0194$) in the DMH (Figure 30 C). No statistically significant differences were detected in the LH (Figure 30 A) and in the PeF (Figure 30 B).

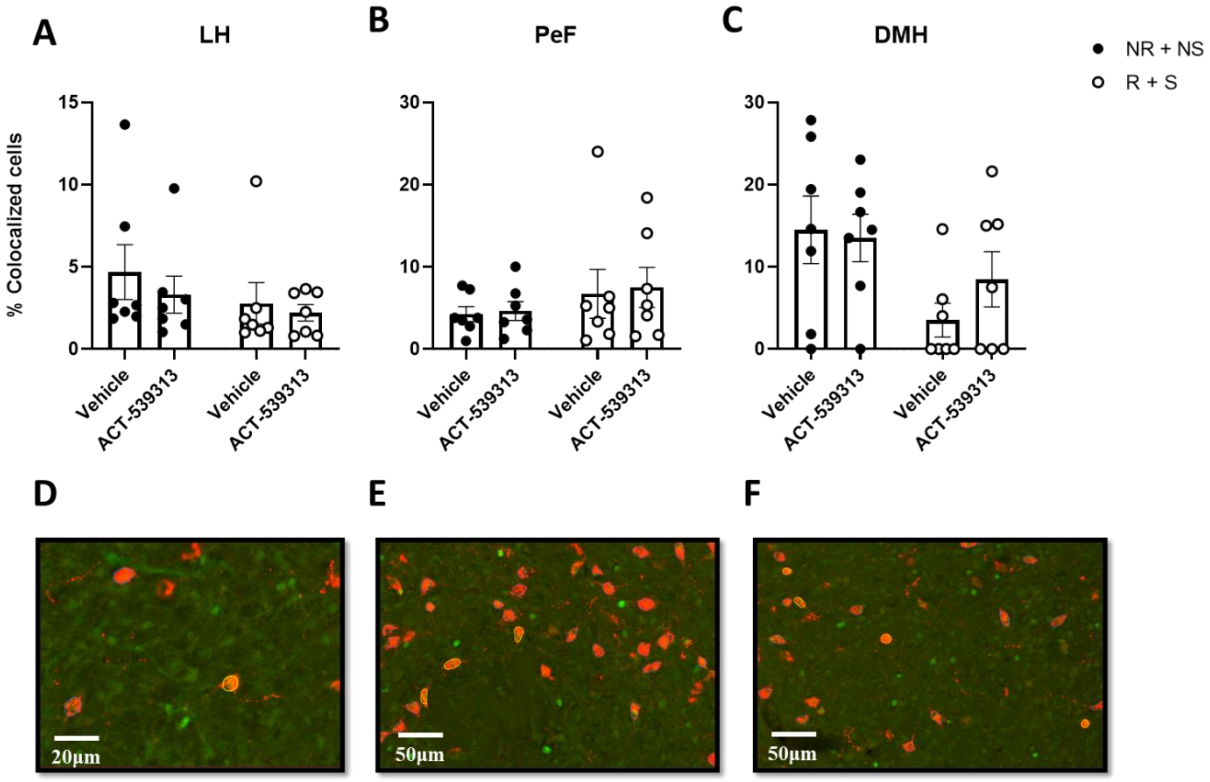


Figure 30. Effect of chronic exposure to the binge eating protocol and chronic treatment with ACT-539313 on the percentage of colocalized cells in the LH, PeF and DMH. The graphs show the percentage of colocalized cells in the LH (A), PeF (B) and DMH (C) of NR + NS and R + S rats chronically treated with vehicle or ACT-539313 (15 mg/kg). The colocalization of delta FosB- and OXA-positive neurons was determined by initially identifying the OX-A positive cells, using the cell detection method, and then applying the pixel classifier for delta FosB, specifically on these neurons. The neurons that were positive for both OX-A-cell detection and delta FosB-pixel classifier were considered colocalized. The colocalization was expressed in terms of percentage of colocalized cells with respect to the total number of OX-A positive neurons. (D, E, F) Representative pictures showing

the identification of the colocalized cells in the LH, PeF and DMH. The ordinary two-way ANOVA test, which included the between-subjects factors of "Condition" (NR + NS vs R + S) and "Treatment" (Vehicle vs ACT-539313 15 mg/kg), followed by the Tukey's post-hoc test to correct for multiple comparisons was used to compare the percentage of colocalized cells in vehicle- or ACT-539313-treated R + S and NR + NS rats. Data are expressed as mean ± SEM. DMH, dorsomedial hypothalamus; LH, lateral hypothalamus; NR + NS, non-restricted plus non-stressed; PeF, perifornical area; R + S, restricted plus stressed; SEM, standard error of the mean.

4.4. Discussion

The aim of the current investigation was to analyze potential changes in OX-A protein expression in multiple brain regions of rats chronically exposed to a well-validated animal model of binge eating (Cifani et al., 2009). In a previous work using the same preclinical model, our group showed that administration of a SO1RA is effective in blocking the binge eating episode in female rats (Piccoli et al., 2012). Interestingly, a similar effect was observed after administration of a dual OXRs antagonist, but not with a selective OX2R antagonist, suggesting that the ability of OX neurotransmission in driving binge eating behavior is predominantly mediated by the OX1R (Piccoli et al., 2012). In this study, another potent and selective SO1RA, named ACT-539313, was tested in the same animal model. The results obtained from the behavioral experiments performed are in line with previous studies in which OX1R antagonists were effective in blocking binge-like eating of palatable foods in both rats and mice (Alcaraz-Iborra et al., 2014; Olney et al., 2015; Piccoli et al., 2012; Rorabaugh et al., 2014; Valdivia et al., 2014). The dose of ACT-539313 used in this work (15 mg/kg) was selected on the basis of a previous dose-response study in the same binge eating model, where it attenuated the compulsive consumption of palatable food selectively in bingeing rats, withouth any effect in the other experimental groups (data not shown;

Idorsia internal report B-18.065). In the present study, the behavioral effect of this compound was further examined under a chronic administration regimen (10 days). Our group has recently demonstrated that in this binge eating protocol, the compulsive consumption of HPF observed on day 25 can be repurposed over-time, by exposing the animals to additional cycles of restriction combined with the stress procedure (Pucci et al., 2022). Interestingly, pre-treatment with ACT-539313 was demonstrated effective in blocking binge eating behavior even under chronic administration, suggesting long-term efficacy.

After chronically exposing (or not) the rats to this protocol of binge eating we analyzed, through an immunohistochemical approach, if OX-A and delta FosB proteins could represent markers of long-term neuronal adaptations and plasticity underlying compulsive-like over-eating. Initially, we evaluated potential changes of OX expression in OX-positive fibers at multiple extra-hypothalamic sites that receive neuronal projections from OX-producing cells (Nambu et al., 1999; Peyron et al., 1998). We observed a reduced OX expression in the R + S group of rats, compared to NR + NS group, in the PVT, CeA and DRN, brain regions important for the OX neurotransmission in driving consumption of palatable food. The PVT is highly innervated by OX-A fibers (Kirouac et al., 2005), and has been proposed as an integrative relay in mediating the reward-related communication between the lateral hypothalamic OX-producing neurons and the dorsal and ventral striatum (Matzeu et al., 2014). Accordingly, Meffre et al. found that direct injection of OX-A in the posterior PVT facilitates cue-driven reward seeking for sucrose in sated rats, and positively modulates the level of cue-evoked responses of NAcC neurons (Meffre et al., 2019). Intra-PVT injection of OX-A increases dopamine levels in the NAc, and the selective knockdown of PVT OX1Rs decreased hedonic high fat feeding in rats (Choi

et al., 2012). The OX1R-expressing neurons in this brain region are also activated by cues associated with food reward (Choi et al., 2010). Thus, these findings, together with the results obtained in our investigation support a relevant role for OX neurotransmission in the PVT in driving non-homeostatic consumption of HPF.

Regarding the CeA, OX-A has been demonstrated to increase the firing rate of neurons of this brain region, via an OX1R-mediated mechanism, and to consequently affect emotional-related behaviors of rodents (Pan et al., 2020). Furthermore, selective administration of OX-A in the CeA was observed to increase consumption of HPF, without any effect on chow feeding (Jin et al., 2020). This suggests that CeA OX-signaling is implicated in the hedonic rather than in the homeostatic aspect of eating behavior. In a previous work by our laboratory, it was also demonstrated that rats exposed to a history of food restriction plus stress (binge eating group) had an increased activation of neurons in the CeA (assessed by c-Fos quantification) (Romano et al., 2020). Thus, it can be hypothesized that OX-A in the CeA triggers binge-like consumption of palatable food and that chronic exposure to this binge eating protocol might result in a down-regulation of OX-neurotransmission in this brain region.

A decrease in OX-fibers was also observed in the DRN of R + S rats. OX-A is known to have a direct excitatory effect on serotonin neurons of the DRN (Liu et al., 2002), and this neuronal circuitry seems to have potential implications in the field of obesity and energy balance (Mavanji et al., 2022). OX-A was additionally reported to exert arousal promoting activities via DRN serotonin neurons (Yang et al., 2019). Considering that these neuronal populations are implicated in binge eating behavior (Cao et al., 2014), and that in previous works we observed an increased general arousal in R + S rats (Cifani et al., 2020; Micioni Di Bonaventura, Lutz, et al., 2017), it could

be hypothesized a potential interaction of OX-A and serotonin in this behavioral effect, and possibly, in driving the over-consumption of HPF in bingeing rats.

Generally, in these brain regions, the decrease in OX-positive fibers in the R + S rats might reflect a down-regulation of the OX neurotransmission in response to repeated exposure to the binge eating protocol. This finding has been previously reported in two studies in which repetitive episodes of sucrose and saccharine binge-like drinking led to a reduction of OX mRNA expression and OX immunoreactivity (Alcaraz-Iborra et al., 2014; Olney et al., 2015), and it might represent a protective neurochemical mechanism against over-consumption of HPF. No statistically significant differences were obtained in the other brain regions considered, the mPFC, the BNST, the LC and the VTA.

The second target of the immunohistochemical analysis was the transcription factor delta FosB, a marker of long-term neuronal adaptation and plasticity (Nestler, 2015; Nestler et al., 2001; Nestler et al., 1999). A recent study reported that binge eating-prone rats revealed higher levels of delta FosB in reward- and stress-related brain regions, compared to binge eating-resistant rats (Quansah Amissah et al., 2020). In contrast, in our work, we were not able to reproduce these findings, observing no significant differences in the expression of delta FosB in any of the brain regions evaluated in binge eating rats compared to the control counterpart. This might reflect the different conditions used in the binge eating protocols. In our model we elicited the episode of binge eating using a frustration mild stress procedure (Cifani et al., 2009), meanwhile in Quansah Amissah et al.' study, three stress sessions were used, each one consisting of four foot-shocks. Probably, our conditions were not intense enough to trigger an increased delta FosB expression in the brains of R + S, or

alternatively, more cycles of restriction and refeeding followed by stress would be needed to elicit changes in delta FosB transcription.

In the last experiment, the three main hypothalamic sites, where OX neurons reside, were analyzed: the LH, PeF and DMH. Experimental evidence suggests that exposure to palatable food on schedules that induce binge-like eating in animals, might trigger the activation of OX neurons, which in turn could result in the hyper-responsivity to food-related stimuli (Buczek et al., 2020; Choi et al., 2010; Freeman et al., 2021; Valdivia et al., 2015). Moreover, hypothalamic OX mRNA levels are up-regulated after binge-like eating of a highly palatable sugar diet (Olszewski et al., 2009), while repetead episodes of sucrose or saccharin binge-like drinking lead to a reduction of OX-A mRNA levels and OX immunoreactivity in the LH (Alcaraz-Iborra et al., 2014; Olney et al., 2015). However, in contrast to our expectation, no statistically significant differences in the number of OX-positive neurons were observed among R + S and NR + NS rats in all three hypothalamic sub-regions analyzed in this study. It is possible that the lack of differences in the amount of OX-positive neurons in the hypothalamus of binge eating and control rats might reflect the timing chosen for the animals' sacrifice, that was performed 24 h after the last binge eating episode. Indeed, some works reported a decrease in OX gene expression immediately following consumption of palatable food (Alcaraz-Iborra et al., 2014; Olney et al., 2015), while others have observed that OX gene expression and OX neuronal activation are increased between 30 min and 2 h after the cessation in palatable food bingeing (Furudono et al., 2006; Olszewski et al., 2009; Rorabaugh et al., 2014; Valdivia et al., 2015; Valdivia et al., 2014). Thus, the expression of OX-A could have returned to basal levels on the day following the last exposure to HPF.

An additional objective of the current investigation was to analyze the effect of chronic administration with the SO1RA ACT-539313 on OX-A and delta FosB expression. We observed that there was no effect of the treatment in the expression of these two proteins in all of the extra-hypothalamic brain region analyzed. However, when we focused on the hypothalamus, a statistically significant effect of "Treatment" in the LH and in the PeF, and a trend for significance in the DMH were observed. The rats chronically treated with ACT-539313 had more OX-positive neurons compared to vehicle-treated rats, suggesting the possibility of a compensatory mechanism by which OX-A expression is enhanced in response to the chronic administration of a SO1RA.

Analyzing changes in the transcription factor delta FosB in the hypothalamus, we were able to find a statistically significant effect of the treatment in the PeF, and a trend for significance in the LH, with rats treated with ACT-539313 showing increased levels of delta FosB in these hypothalamic sites. In the brain, transcription of delta FosB can be induced by chronic pharmacological treatments, including antidepressants and antipsychotic, and by repeated administration of drugs of abuse (Brenhouse & Stellar, 2006; Dietz et al., 2014; Nestler, 2015; Perrotti et al., 2008). Thus, it is possible that chronic daily administration of the SO1RA ACT-539313 might have led to an increased transcription of delta FosB in part of the hypothalamic neurons. However, a very low degree of colocalization of OX-A and delta FosB was observed in OX-expressing neurons. This suggests that chronic injection of ACT-539313 could possibly activate populations of neurons different from OX-expressing cells, even though this needs to be evaluated in future studies.

Finally, considering the study of Vialou et al., in which OX signaling is required for the ability of delta FosB over-expression to increase instrumental responding for sucrose (Vialou et al., 2011), we evaluated the degree of colocalization of delta FosB

within OX-expressing neurons. To date, only one study (Flores et al., 2014) investigated the possible activation of OX neurons using double label immunofluorescence of OX-A and delta FosB, reporting that self-administration of the cannabinoid agonist WIN55,212-2 increased the percentage of OX-cells expressing delta FosB in the LH. However, in this study, the percentage of colocalized cells with respect to the total number of OX neurons was relatively low in all three hypothalamic sites. Thus, in this animal model of binge eating, we were not able to detect a statistically significant interaction of OX-A and delta FosB in mediating binge eating behavior.

In conclusion, this study supports the potential use of SO1RAs in the treatment of binge eating behavior, whose efficacy might be maintained not only after acute administration but also in the long-term period, under chronic administration regimens. However, a compensatory increase in OX-expression is observed under daily administration of ACT-539313. Binge eating behavior also correlates with alterations in the OX-A protein expression at extra-hypothalamic brain regions, such as the PVT, CeA and DRN, possibly reflecting a down-regulation of OX-signaling in regions implicated in the over-consumption of palatable foods.

5. CONCLUSION

BED is the most common eating disorder, representing a major causal factor for obesity, and an independent risk factor for several metabolic, physical and psychiatric disorders. Despite the high frequency of this eating disorder (more commonly observed during adolescence), BED is still under-recognized and under-treated. Psychological approaches, generally recommended by international guidelines, are not

always successful in the management of BED. Consequently, pharmacological therapies are necessary for the treatment of this psychiatric disorders, Currently, the only approved drug is LDX, an inactive prodrug of d-amphetamine, which is effective in improving binge eating symptoms in BED individuals, but it is also associated to multiple side effects, including risk of abuse and dependence, anxiety, and increased heart rate and blood pressure. Thus, the research for new compounds effective in the treatment of BED and well-tolerated by patients is necessary and urgently needed. In this context, animal models offer the opportunity to investigate the neurobiological processes underlying binge eating behavior, and represent an important tool to test the efficacy of innovative pharmacological strategies. The preclinical binge eating model discussed in this work, developed by Cifani et al. in 2009, is characterized by different elements of face validity, which include the presence of cycles of caloric restriction and stress, two conditions that are frequently observed in BED individuals and that might increase in the risk for binge eating episodes in the general population. Another element of face validity is the use of HPF, resembling the "forbidden" foods by human binge eaters. This animal model has been also characterized by a high degree of predictive validity, since drugs that act by suppressing appetite were demonstrated to non-selectively reduce the HPF intake, under both binge and non-binge conditions. Differently, drugs like topiramate, frequently used off-label for BED, were found to selectively attenuate consumption of HPF in the binge eating group of rats.

In the present experimental thesis, using this animal model of binge eating, it was provided evidence that σ1R antagonists and SO1RAs might represent important and innovative pharmacotherapies for bingeing-related eating disorders.

The σ1R antagonist evaluated, characterized by an impressive selectivity for the σ1R over the σ2R subtype, was able to block the binge eating episode selectively in rats

with a history of food restriction exposed to the stress procedure. Given that this compound did not affect anxiety- and depressive-like behaviors in rats, it is possible that blocking the σ1R represents a strategy to selectively target the neurobiological mechanisms driving compulsive consumption of HPF, without influencing other aspects of animals' behavior or psychological conditions.

In the same animal model, we also demonstrated that SO1RAs are able to block the binge eating episode, and this occurs both under acute and chronic administration regimens, suggesting no development of tolerance during long-term treatments. OXs are powerful stimulator of food intake under certain enviornmental conditions, and they can promote binge-like consumption of palatable foods. Here, we have demonstrated that chronic exposure to a binge eating protocol might lead to compensatory down-regulation of OX-A protein expression at extra-hypothalamic sites, such as the CeA, the DRN and the PVT. This could reflect an adaptation of the OX system, and might represent a protective neurochemical mechanism, preventing subsequent overconsumption of palatable foods.

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