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"Behavioral and neuronal effects induced by appetite suppressants: modulation of the nucleus accumbens activity in rats"

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Abbreviations

ADR - Adrenaline

AMPH - Amphetamine

BMI - Body Mass Index

CCK - Cholecystokinin

CDC - Centre for Disease Control

DA - Dopamine

DALYs - Disability Adjusted Life Years

DEP - Diethylpropion hydrochloride

EMEA - European Medicines Agency

FDA - Food and Drug Administration

5-HT - Serotonin

i.g. - Intra-gastric

i.p. - Intraperitoneal

LFP´s - Local Field Potentials

NAASO- North American Association for the Study of Obesity

NA - Nor-adrenaline

NAc - Nucleus accumbens

NHLBI - National Heart Lung and Blood Institute

NIDDK - National Institute of Diabetes and Digestive and Kidney Institute

NORP - d-Norpseudoephedrine hydrochloride

NCD - Non-Communicable Diseases

NIH - National Institute of Health

PCA - Principal Components Analysis

PHEN - Phentermine hydrochloride

PYY - Peptide YY

SSRI's- Selective Serotonin Reuptake Inhibitors

VLCD - Very Low Calorie Diet

WHO - World Health Organization

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Resumen

La obesidad es un problema de salud que ha alcanzado proporciones epidémicas a nivel mundial. Una manera de enfrentar este problema es con el uso de coadyuvantes farmacológicos supresores del apetito. Estos compuestos son con frecuencia derivados de la anfetamina (AMPH) tales como el dietilpropión (DEP), fentermina (PHEN) y la D-norpseudoefedrina (NORP) cuyos efectos están mediados a través de la modulación de los niveles de serotonina, noradrenalina y dopamina. El núcleo accumbens shell (NAc-shell) recibe entradas dopaminérgicas y participa en la alimentación y la actividad motora. Sin embargo, poco se sabe acerca de cómo los supresores del apetito modulan su actividad. Por lo tanto, en esta tesis caracterizamos los efectos del tratamiento a corto plazo con DEP, PHEN y NORP sobre la conducta y la actividad neuronal del NAc shell. Encontramos que la administración periférica de estos compuestos causó una disminución transitoria en la ingesta de alimentos y el peso corporal mientras que como efectos secundarios incrementó la locomoción, estereotipia e indujo insomnio, así como provocó un desbalance inhibitorio en la actividad poblacional del NAc shell. Este desbalance correlacionó con el inicio de la locomoción y la estereotipia. El análisis de los potenciales de campo locales (LFP´s de sus siglas en inglés) mostró que los tres fármacos también modularon las oscilaciones beta, theta y delta registradas en el NAc shell. Estas oscilaciones no reflejan un estado cerebral de aversión o malestar como lo demuestran las observaciones basadas de experimentos de aversión al sabor, pero si muestran una correlación tanto en la disminución inicial en la ingesta de alimentos y el peso como en la subsecuente tolerancia a estos fármacos. Es importante destacar que la pérdida de peso y la locomoción, inducidas por la administración de los fármacos, se redujeron notablemente tanto por la administración intragástrica como por su administración directamente en el NAc shell, de antagonistas de los receptores de dopamina SCH23390 (D1-receptor) o raclopride (D2-receptor). Además, ambos antagonistas revirtieron las oscilaciones del LFP y restauraron parcialmente el desequilibrio en la actividad del NAc shell inducidas por el supresor del apetito. Estos datos revelaron que el efecto supresor del apetito y la actividad neuronal registrada en el NAcshell inducidos por la administración de DEP, PHEN y NORP depende en gran medida de la activación dopaminérgica lo cual demuestra que los receptores tipo D1 / D2 (en el NAC shell) desempeñan un papel importante en el mecanismo de acción de estos compuestos anoréxicos.

Abstract

Obesity is a worldwide health problem that has reached epidemic proportions. To ameliorate this problem one approach is the use of pharmacologic appetite suppressants. These compounds are frequently amphetamine (AMPH) congeners like diethylpropion (DEP), phentermine (PHEN) and D-Norpseudoephedrine (NORP) whose effects are mediated through serotonin, norepinephrine and dopaminergic pathways. The nucleus accumbens shell (NAc-shell) receives dopaminergic inputs and is involved in feeding and motor activity. However, little is known about how appetite suppressants modulate its activity. Therefore, we characterized behavioral and neuronal NAc-shell responses to short-term treatments of DEP, PHEN and NORP. These compounds caused a transient decrease in weight and food-intake while increasing locomotion, stereotypy and insomnia and evoked a large inhibitory imbalance in NAc-shell spiking activity that correlated with the onset of locomotion and stereotypy. Analysis of the local field potentials (LFPs) showed that all three drugs modulated beta, theta and delta oscillations. These oscillations do not reflect an aversive-malaise brain state as ascertained from taste aversion experiments, but tracked both the initial decrease in weight and food intake and the subsequent tolerance to these drugs. Importantly, the appetite suppressant-induced weight loss and locomotion were markedly reduced by intragastric (and intraNAc-shell) infusions of dopamine antagonists SCH23390 (D1-receptor) or raclopride (D2-receptor). Furthermore, both antagonists attenuated appetite suppressant-induced LFP's oscillations and partially restored the imbalance in NAc-shell activity. These data revealed that appetite suppressant-induced behavioral and neuronal activity recorded in the NAc-shell depend on to various extents on dopaminergic activation thus point to an important role for D1/D2-like receptors (in the NAc-shell) in the mechanism of action for these anorexic compounds.

1. INTRODUCTION

1.1 Obesity

Obesity was defined as an epidemic of the 21st century by the World Health Organization (WHO) and has become a serious health problem worldwide. Excess caloric intake and too little physical activity contribute the most to obesity, but of course genetic susceptibility and various eating disorders may also contribute (McPherson 2014). The WHO and the USA National Institutes of Health (NIH) have defined obesity as excessive or abnormal body fat accumulation that drives a risk to health and it is diagnosed base on the Body Mass Index (BMI), with a BMI of 25–30 defined as overweight and a BMI >30 classified as obese (WHO, 2008). It has been estimated that at least 3.4 million people die each year as a result of being overweight or obese and 35.8 million (2.3%) of global Disability Adjusted Life Years (DALYs) are caused by this pathology. DALY is a health gap measure that extends the concept of potential years of life lost due to premature death to include equivalent years of “healthy” life lost by virtue of being in states of poor health or disability.

1.2 Prevalence of Obesity

The prevalence of overweight and obesity were highest in the WHO Regions of the Americas (62% for overweight in both sexes, and 26% for obesity) and lowest in the WHO Region for South East Asia (14% overweight in both sexes and 3% for obesity). Women's obesity was significantly higher than men’s, with the exception of high-income countries where it was similar. In low and lower middle income countries, obesity among women was approximately double that among men (WHO, 2008). In 2012, according to the Mexican National Health Survey and Nutrition (ENSANUT) in over 20 years prevalence of overweight was higher in men (42.56%) than in women (35.48%). The prevalence of obesity was higher in women (37.5%) than in men (26.8%), but by adding the prevalence of overweight and obese women 73.01% and 69.37% of men have combined prevalence of overweight or obese.

The worldwide prevalence of obesity nearly doubled between 1980 and 2008, and it currently affects approximately 30–35% of the general population in the USA and 25% in the UK. (Rodgers et al. 2012; Wang and Beydoun 2007). Moreover, childhood obesity is currently one of the most serious health challenges, and its prevalence has increased worldwide at an alarming rate in recent decades (Capobianco et al. 2009; Carotenuto et al. 2006). In 2012, more than 40 million children under the age of five were overweight or obese. Additionally, 70 million children under age five will be overweight or obese by 2025 if current trends continue. The WHO has estimated that in 2035 more than 300 million adults and children will be obese.

Mexico is not the exception to this global trend, since it has experienced a rapid increase in wealth in recent era, which brings a significant shift in socio-economic status and a geographic shift from rural to urban among its population. This has led to changes in diet that are harmful to health: an increase in physical inactivity and increased access to low-priced highly energy-dense foods. As a result, rapid growth in the prevalence of obesity and obesity-related non-communicable diseases (NCD) has been observed with a lack of preventive steps to curb this rise.

1.3 Obesity and its consequences

The ancestors of humans experienced situations that contrasted with the present environment, which was characterized by availability of abundant food and low physical exercise (i.e. obesogenic environment). These changes have converted an evolutionary benefit into a serious metabolic problem and obesity (Haslam and James 2005). Obesity is associated with increases in morbidity, premature mortality, impaired quality of life, and large health care cost (Fontaine et al. 2003; Haslam and James 2005; Kopelman and Grace 2004; Lawrence and Kopelman 2004). The major comorbidities include the following: metabolic syndrome, type 2 diabetes, hypertension, dyslipidemia, myocardial infarction, and certain cancers (Carotenuto et al. 2012; Flegal et al. 2007). Obesity is associated with increases in morbidity, premature mortality, impaired quality of life, and large health care cost.

1.4 Basic concepts of ingestive behavior

The term ingestive behavior describes all the features of the relationship between animals (including humans) and food. This relationship was characterized by the physiological, psychological and social forces that are integrated in the brain and determine not only how much we eat, but also what, when, and how we eat.

Body weight control consists of a complex mechanism regulated by hormonal, metabolic, and nervous pathways. An effective treatment for obesity would require an efficient knowledge of factors and mechanisms potentially regulating food intake, energy balance, and body fat mass deposition (Sharma and Padwal 2010). Understanding the heterogeneity of the condition and the underlying pathophysiological mechanisms will aid the development of effective treatments.

Food reward can be understood better if broken down to its appetitive and consummatory domains. The appetitive reward value of food reflects the effort of the animal or human is willing to exert in order to obtain it (e.g. how much do I want this chocolate cake?). The consummatory reward value of food reflects the “pleasure” elicited when food is actually consumed (e.g. how much do I like this chocolate cake?). Indeed, appetitive behavior is thought to be principally determined by dopamine neurotransmission (Berridge 2007) and consummatory behavior determined by opioid (Pecina and Berridge 2005; Zhang et al. 1998) and endocannabinoid (Higgs et al. 2003) neurotransmission in animals. Ingestive behavior is not only affected by physiological signals, but also by psychological traits and social influences. In this section some of the potential causes are explored with a particular emphasis on the physiological mechanisms that are associated or contribute to weight gain and disordered eating behavior.

1.4.1 Homeostatic control of food intake

The homeostatic control of food intake takes place predominantly within the hypothalamic region that communicate through several neurotransmitters (Benarroch 2010). This nucleus responds to signals originating from the presence of nutrients in the gut and fat stores (such as ghrelin and leptin, respectively see below).

The activation either increases or decreases food intake both during a meal but also more chronically in an attempt to keep body weight stability in the long term.

The **lateral hypothalamus** contains two different groups of cells that produce orexin (hypocretin) and melanin concentrating hormone (MCH) that communicate with the arcuate, other hypothalamic areas, brainstem and the mesolimbic system to control food intake. Lesion studies provided the first clue on the importance of the medial hypothalamus in the control of food intake. Lesions of the medial hypothalamus caused obesity and of the lateral hypothalamus weight loss, suggesting that the former is the satiety and the latter the feeding center (King 2006). Since these initial descriptions, hypothalamic anatomy and function have been further refined. The arcuate nucleus of the hypothalamus contains two groups of neurons with opposite effects (van den Top and Spanswick 2006). The first group synthesize pro-opiomelanocortin (POMP) derived peptides, amongst which α-melanocyte stimulating hormone acts via the melanocortin 4 receptors on the paraventricular nucleus, lateral hypothalamus and the ventromedial nucleus to lower food intake and increase energy expenditure (Millington 2007). The second group of neurons synthesize neuropeptide Y (NPY), agouti-related protein and gamma-aminobutyric acid (AGRP) which increase food intake and lower energy expenditure by inhibiting POMC neurons, but also by projecting to the paraventricular, ventromedial, dorsomedial nuclei and lateral hypothalamus via Y1, Y2 and Y5 receptors and antagonism of α-melanocyte stimulating hormone at melanocortin 4 receptors (Meister 2007). Moreover, using optogenetic tools and transgenic Cre-mice to definitely confirm that the selective activation of AGRP/NPY neurons and its downstream outputs are sufficient to evoke voracious eating without previous training. They found that AGRP neuron–mediated feeding was not dependent on suppressing this melanocortin pathway, indicating that AGRP neurons directly engage eating circuits (Aponte et al. 2011).

The orexin neurons are active during fasting, inactive during sleep and inhibited by glucose (Tsujino and Sakurai 2009). They act through orexin 1 and 2 receptors to regulate arousal, stress responses but also to alter brain reward systems, particularly dopaminergic (DA) function, through their effects on the ventral

tegmental area (VTA). By contrast, the melanin concentrating hormone group are stimulated by glucose and active during sleep, project to the nucleus accumbens and increase food intake via melanin concentrating hormone 1 and 2 receptors (Guyon et al. 2009).

1.4.2 Hedonic control of food intake

Emotional and cognitive aspects of food or other stimuli are processed in the limbic and cortical areas of the brain. One current theory dictates that obesity arises from alteration of the activity of reward brain areas by down-regulating expression of D2 receptors in striatum (Volkow et al. 2011), which in turn can trigger compulsive eating behavior (Johnson and Kenny 2010).

The **dorsal striatum**, including the caudate and putamen, as part of the reward system, is important in learning through the association of a specific action with their expected reward value, including both food and non-food rewards. This goal-directed behavior is expressed through the release of dopamine in the dorsal striatum in response to a specific cue, especially when hungry (Balleine et al. 2007; Volkow et al. 2002). The release of dopamine in the dorsal striatum in healthy volunteers also correlates with the subjective pleasantness of food in a seminal PET study (Small et al. 2003). In particular, fMRI activation of the caudate (and also hippocampus and insula) correlates with cravings for food when healthy volunteers are asked to imagine the sensory properties of their favorite food (Pelchat et al. 2004).

The **nucleus accumbens (NAc)** is part of the ventral striatum, and responds to both positive and negative stimuli from the environment and determines goal directed behavior (Ikemoto and Panksepp 1999; Kelley 2004). In particular, novel stimuli activate the NAc which in turn initiates seeking behavior and motivation to the stimulus (Ikemoto and Panksepp 1999; Kelley 2004). In healthy adults, differences in reward sensitivity correlate with fMRI activation in the NAc (and also the orbitofrontal cortex, amygdala, midbrain and ventral pallidum) in response to pictures of appetitive foods (Beaver et al. 2006). Successful dieter’s exhibit higher NAc activation in response to appetitive food pictures after a milkshake preload compared

to non-dieters who exhibit higher nucleus accumbens activation after a water preload (Demos et al. 2011). NAc activation to food cues in overweight adults is attenuated by satiation and further this predicts subsequent food intake (Fletcher et al. 2010). Importantly, although some authors have interpreted NAc neuronal inhibitions as encoding reward (Carlezon and Thomas 2009; Roitman et al. 2005). Furthermore,Krause et al. (2010) demonstrated that NAc neurons inhibit palatable food consumption and that a pause in their firing is required to initiate and maintain consumption.

Briefly, NAc responds directly to both the appetitive and consummatory reward of food/taste through DA pathway (Ikemoto and Panksepp 1999). Lesions of VTA DA neurons dramatically impaired free-feeding (Narayanan et al. 2010). In addition, infusion of MCH promotes eating by globally decreasing the firing rate of the NAc shell. Moreover, an MCH antagonist inhibits feeding (Sears et al. 2010). It has been found that global inactivation of NAc shell promotes eating (Taha and Fields 2006). Although we observed that a similar proportion of NAc shell neurons are also excited during free-feeding (Tellez et al. 2012), suggesting that during physiological conditions, feeding behavior induces a balance of excitation and inhibition. Nevertheless, temporary global inactivation of NAc Shell (e.g., by infusing Muscimol) promotes abnormal over-eating in sated rats, similar to that induced by exogenous MCH infusions (Baldo et al. 2004). The opposite effect was evoked after electrical activation of NAc shell since it refrained ingestive behavior (Krause et al., 2010). All these evidence have supported the ‘‘inhibitory gating hypothesis’’ that propose that a general pause in the NAc shell activity is necessary to initiate and maintain ingestive behavior (Krause et al. 2010). More importantly, NAc is a node in the eating circuit also that is directly or indirectly coordinated by the sleep-awake circuitry (Tellez et al. 2012). Hence, the above described literature evidence strongly supports the idea of understanding the neuronal correlates of eating might be relevant to understand the neurobiological basis of obesity, since obesity may induce a hypoactive state or blunted response on the striatum (Stice et al. 2008).

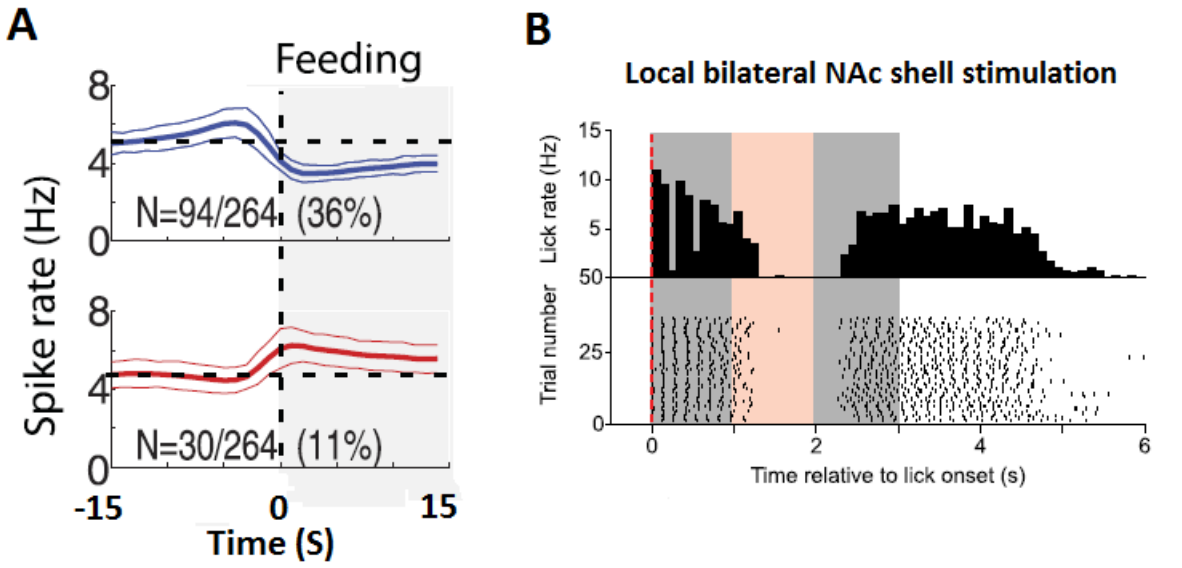

Figure 1. Nucleus accumbens shell (NAc shell) spiking heterogeneity and population activity around eating (Gating feeding hypothesis). (A) Obtained from Tellez et al., 2012, Top: the population PSTH activity of all inactive neurons (blue) with responses aligned to time = 0 S for the licking for Ensure. Bottom panels show the population activity of active neurons for eating. Note that the majority (36%) of NAc shell population activity was inhibited during feeding **(B)** Adapted from Krause et al., 2010. Per-lick interval histograms and lick raster plots of local stimulation for (Orange Shading, 1-2 S). Bilateral local stimulation in the NAc shell disrupts licking for sucrose.

1.4.3 Integration of homeostatic and hedonic systems

The homeostatic and hedonic networks that govern food intake and body weight are not anatomically or functionally distinct but continuously interact. This is facilitated by the functional connections between the hypothalamic, mesolimbic and cortical circuits but also by the peripheral metabolic peptides which exert their effects on targets within these circuits at multiple points in the brain (Kampe et al. 2009). Overall, many previous studies suggest that weight loss leads to a heightened activation of both the homeostatic and reward controls of food intake in such a way as to make long-term weight loss maintenance difficult. The combination of higher cognitive restraint and pharmacological “assistance” in these maintenance efforts may increase the chances of successful outcomes.

The above mentioned brain regions do not act in isolation but are interconnected and signal to each other through specific neurotransmitters. The regulation of ingestive behavior that directly or indirectly convey information to the

brain's (NAc) DA reward pathway with a particular prominent role of the hypothalamus. Hence, from this it is clear that dopamine neurotransmission is important in mediating the appetitive reward value of food in animals (Berridge 1996).

1.5 Treatments of Obesity

1.5.1 Lifestyle Modification

Appetite reduction is a compelling goal for long-term weight loss and resolution of the metabolic effects of obesity and overweight. The abundance of highly palatable food that influences eating patterns can make caloric restriction an unachievable goal for many individuals. In obese patients who have achieved weight loss through lifestyle changes, clinical data suggest the presence of compensatory mechanisms that lead to weight regain in 80-90% of individuals (Sumithran et al. 2011; Wing and Hill 2001). One study reported that overweight and obese patients who underwent a 10-week weight loss program had significantly lower levels of leptin, Peptide YY, Cholecystokinin, insulin, and amylin and significant increases in ghrelin levels from baseline. These differences persisted at one year and were accompanied by significant increases in appetite and preoccupation with food (Sumithran et al. 2011). Acute fasting and chronic dieting have been shown to induce changes both in the hypothalamic and reward areas of the brain, with the net effect of further increasing hunger, and pre-occupation and craving for caloric dense food (Doucet et al. 2000; Goldstone et al. 2009; LaBar et al. 2001).

1.5.2 Surgical

Currently, surgery offers the most effective method to treat morbid obesity. However, the obese patient must first assess carefully considering the risks associated comorbidities (Sjostrom 1992) then consideration should be given adequate levels of therapeutic modalities based on the severity of obesity and its associated risks (Mun et al. 2001). Weight reduction through surgery has resulted in improvements in blood pressure, serum lipid levels, type 2 diabetes, respiratory failure caused by sleep apnea and obesity hypoventilation syndrome, reflux esophagitis and venous stasis ulcers.

Surgical Procedures

Bariatric procedures for weight reduction share two main designs: intestinal malabsorption and gastric restriction. Malabsorption procedures involve rearranging the small intestine, to reduce the efficiency or functional length of the intestinal mucosa to absorb nutrients, while restrictive operations, involving the creation of a small gastric pouch with an outlet, so that the capacity decreases food intake (Mun et al. 2001).

1.5.2.1 Malabsorptive Procedures

Jejuno-Ileal Derivation

The first bariatric surgery to treat obesity was the jejunum-ileum bypass (Payne et al. 1973). This procedure creates a proximal jejunum anastomosis in the terminal ileum. The jejunum-ileum bypass is only a process of malabsorption, stomach is not changed to limit food intake, and do not need to make significant changes in eating habits to induce weight loss. However, this operation was restricted by an unacceptable level of serious complications such as liver failure (Halverson et al. 1978), hepatic cirrhosis, renal oxalate, enteric bypass, arthritis, and multiple metabolic deficiencies, such as protein malnutrition, bone disease metabolic, hypocalcemia, and deficiencies of vitamin B12 and vitamin D.

Biliopancreatic diversion with duodenal switch

Biliopancreatic diversion is primarily a malabsorptive procedure involving divert the bile and pancreatic secretions distal ileum (in humans 50 cm). A small degree of gastric restriction is added by performing a distal gastrectomy. Combining gastro ileostomy, a long bilio-pancreatic tip, and a short common stem channel in poor digestion and poor absorption of nutrients significant. This procedure is very effective in inducing weight loss, particularly in obese patients "super morbid" (BMI> 50 kg/m^2). However, it is also important to note that this procedure may present further complications such as protein malnutrition, metabolic bone disease, and deficiencies in fat-soluble vitamins, iron, calcium and vitamin B12 (Murr et al. 1999).

1.5.2.2 Restrictive procedures

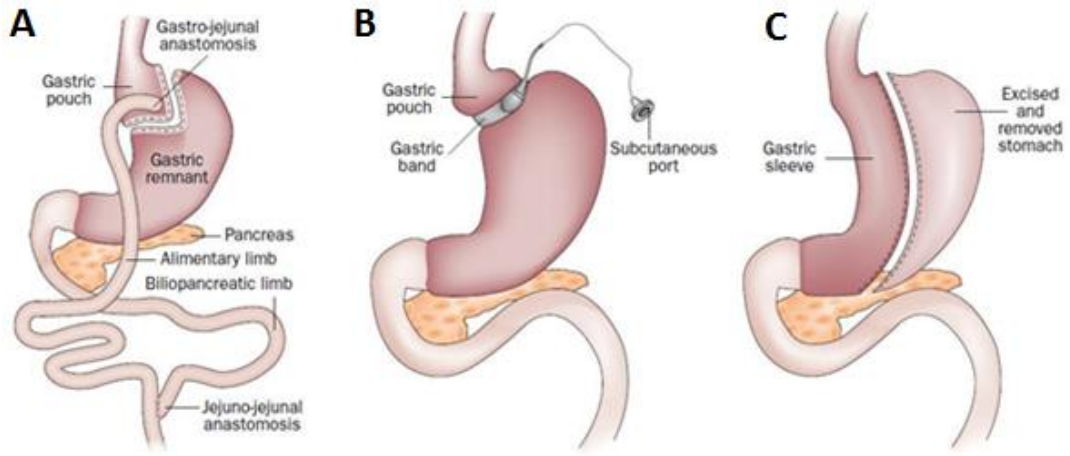

Figure 2. Anatomical changes in the most commonly performed bariatric surgery procedures. (A) Roux-en-Y gastric bypass (RYGB), **(B)** Adjustable gastric banding (BAND), **(C)** Vertical sleeve gastrectomy (VSG).

Gastroplasty

The gastroplasty restricts the storage capacity of the stomach to decrease the ability to consume solid food. Initially, horizontal gastroplasty involved dividing the stomach into a small pouch proximal and distal remnant a large bag, which is communicated through a narrow channel or stoma. Later the procedure was modified to a vertically oriented (Mason 1982) gastroplasty. However, this procedure has been reported a high incidence of stenosis (MacLean et al. 1993).

Gastric Banding

The gastric band is another restrictive procedure that surrounds the proximal stomach with adjustable silicone prosthetic device to divide it into two parts. This procedure is indicated for obese people who have a BMI> 40 kg/m^2. (Bo and Modalsli 1983). Although the results of the gastric band are highly variable, they can be compared with those observed in the vertical banded gastroplasty (Figure 2B).

Gastric bypass

Gastric bypass is primarily a restrictive operation that creates a small gastric pouch and a narrow stoma in order to inhibit the patient's ability to ingest a large portion of food (Figure. 2A). The most common form of gastric bypass surgery called

Roux-en-Y (RYGB) anastomosis, which is named after the surgeon who described it (César Roux) (Sugerman et al. 1996). The procedure is called a referral because the joint between the abdominal pouch and the small intestine is moved to a position below the stomach where food normally passes into the small intestine. The result is that the food passes through a much smaller part of the small intestine, so that the amount of calories and nutrients are absorbed is reduced.

In summary, as described above there are a variety of surgical methods for treating obesity methods, although they have proven to be highly effective (Kral and Naslund 2007). Due to the high cost, and because not all patients are candidates for these procedures, their use is impractical to meet the needs of an entire population, Against this background, it is imperative to conduct basic research to understand the mechanism of action of different anorexigenic currently used for weight loss. Therefore, in the study we have characterized the anorexigenic activity of a drug that has been used for over 50 years but which there is a dearth of information on the mechanism and site of action.

1.5.3 Pharmacological treatment for obesity

The development of effective drug therapies to treat obesity has been both one of the greatest hopes and one of the biggest disappointments (Kushner 2014). As above reviewed, obesity is a complex disorder which has been linked to disturbances in metabolism and behavior, with these disorders are able to disturb the normal mechanisms of regulation of body weight. Logically, both metabolic and behavioral components may be susceptible to drug treatment, made by various agents which have been developed to modify specific points such as; the appetitive and ingestive behavior, the amount of food intake, satiety, nutrient uptake, energy expenditure, among others (Kaplan 2005). Drug treatment for obesity therapy is primarily aimed weight loss, maintenance of this loss and risk reduction (Gutierrez, 2012,).

Drug therapy should be considered in obese and overweight patients with a BMI greater than 27 kg/m^2, especially when these patients have comorbidities such as type 2 diabetes, hypertension, large waist circumference, or when regimes in

eating habits and set workout routines have not yielded results in the desired weight loss (NIH, 2000).

1.5.3.1 Drugs that reduce food intake (anorexigenic)

Most prescription drugs for obesity regulate satiety through an effect on serotonergic (5-HT), DA systems in the hypothalamus (Clapham et al. 2001) noradrenergic receptors. This allows reducing appetite or hunger and, therefore, decreased food seeking behavior.

Noradrenergic

Amphetamine (AMPH) and similar drugs phentermine, diethylpropion, phendimetrazine, benzphetamine, fenproporex, clobenzorex and phenylpropanolamine, indirectly acting sympathomimetic agents such as increasing the release of catecholamines (in areas of the brain involved in ingestive behavior). For example it inhibits DA reuptake in nerve terminals in the paraventricular nucleus of the hypothalamus (Cerulli et al. 2007), which results in stimulation of α1, β1 and β2 adrenergic receptors and therefore reducing appetite (Clapham et al. 2001). Phentermine and diethylpropion are FDA-approved as an anti-obesity treatment for short-term use. These drugs has been available as a monotherapy for obesity since the beginning of ~1960. Its use it is limited to 3 months maximum and recommended establishing a restriction in dietary calories (Goldstein and Potvin 1994).

None of these anorectic drugs has been approved for extended pharmacotherapy. Since stimulation of activity in the sympathetic nervous system may cause an increase in heart rate, heart contractility, cardiac output, conduction velocity, and vasoconstriction effects of adrenaline (ADR), and DA. Besides chronic activation of the sympathetic nervous system may contribute to the pathogenesis of myocardial ischemia and heart failure (DeWald et al. 2006). Other side effects of noradrenergic drugs include nervousness, anxiety, insomnia, dry mouth, sweating, nausea, constipation, euphoria and hypertension (Bray 2000).

Serotonergic

5-HT mimetic agents trigger activities that modify the activity of serotonin in their hypothalamic receptors (5-HT 1B and 5 HT2C), which are involved in food intake (Smith et al. 1999). Specifically, these agents suppress appetite and promote body weight loss by stimulating the release of central and peripheral 5-HT and / or inhibition of neuronal reuptake of 5-HT in the hypothalamus-pituitary-adrenal axis. These drugs include fenfluramine, its dextrorotatory stereoisomer dexfenfluramine, fluoxetine and sertraline, or several serotoninergic precursors, such as 5-HTP.

Unlike fenfluramine, which triggers the release of 5-HT, fluoxetine and sertraline are selective serotonin reuptake inhibitors (SSRIs) approved for the treatment of anxiety and depression (Mayer and Walsh 1997).

Fenfluramine and dexfenfluramine, were widely used to treat obesity until 1997 when they were voluntarily removed from the market due to reports of significant adverse effects including primary pulmonary hypertension (Connolly et al. 1997).

Serotonergic-noradrenergic

Sibutramine is a tertiary amine which acts through its active metabolites (secondary and primary amine) produced from hepatic demethylation. These metabolites of centrally acting α1 and β1 adrenergic receptors and serotonin 5-HT2A and 5-HT2C, inhibiting the reuptake of both 5-HT and NA and also effects on DA (Bray and Greenway 1999).

Its mechanism of action is twofold: on the one hand the increased availability of 5-HT to affect their release and reuptake of serotonin in the synaptic cleft and direct activation of 5-HT receptor reduces food intake and otherwise it stimulates thermogenesis due to stimulation by increasing energy expenditure.

The serotonin 5-HT1B and 5-HT2C are specifically recognized as mediators of 5-HT to induce satiety, probably acting via the hypothalamic melanocortin system. Acting through 5-HT1B, 5-HT and inhibits hyperpolarizes NPY / AGRP neurons, thus reducing the inhibitory GABAergic (gamma-Aminobutyric acid) cells stimulation of

proopiomelanocortin (POMC). Serotonergic agents also activate POMC neurons in the hypothalamus through 5-HT2C receptors which then activates the melanocortin receptor 3 and 4 resulting in the reduction of food intake (Heisler et al. 2006).

Sibutramine belongs to this class of agents known as SNRIs (reuptake inhibitors of NA and 5-HT) and were used majorly in USA as a new class of agents for treating obesity until 2010 (Colman 2005). However, it has recently been withdrawn from the market in some countries due to the incidence of cases reported cardiovascular side effects. Within these agents also is duloxetine and venlafaxine.

1.5.3.2 Drugs affecting the metabolism

An important component of obesity is the intake of food high in fat, this diet provides the body with a lot of calories and low in nutrients (Golay and Bobbioni 1997). Calorie controlled diets and / or restricted fat provide the best means to limit the total energy consumption, but the long-term success of this strategy is often affected by the drop-out of patients from the nutritional regimen. Currently several compounds available are capable of interfering with the metabolism of nutrients before absorption occurs.

Pre-absorptive

The first authorized in Spain and Europe for inhibiting fat absorption drug was orlistat or tetrahydrolipstatin, this drug was first isolated from a soil bacterium (*Streptomyces toxytricini*) and was approved in 1998 for human use. Orlistat inhibits lipase to join them in the intestinal lumen and preventing the cleavage of triglycerides into free fatty acids and mono-glycerides. Thus the absorption of 30% of ingested fats, which are eliminated with the faces is prevented, however, the absorption of fat soluble vitamins (A, D, E, K) are also difficult and gastrointestinal side effects may occur as its action is local (Guerciolini 1997). In addition, this drug exhibits therapeutic activity locally in the lumen of the stomach and small intestine and does not significantly affect systemic absorption. Metformin was not as effective as the lifestyle intervention in the DPP, requiring treatment of 13.9 individuals for 3 years to prevent one case of diabetes, but it was particularly effective in younger individuals and in those who were overweight (Salpeter et al. 2008).

Post-absorptive

Metformin produces anti-hyperglycemic activity by decreasing hepatic glucose production (by decreasing gluconeogenesis) and increasing peripheral insulin sensitivity and apparently promotes weight loss, specifically involving the adipose tissue, although the range of weight loss varies widely among patients (Guerciolini 1997).

1.5.3.3 Drugs that increase energy expenditure

Another strategy for treating obesity involves increasing energy expenditure increased by pharmacological agents that stimulate thermogenesis. Thermogenesis can be achieved by central activation of the sympathetic nervous system or peripheral stimulation of β3 atypical receptors (Astrup et al. 1992). Among the agents that produce ephedrine with caffeine thermogenesis, thyroid hormone, and β agonists (Bray and Greenway 1999) they are included. The only therapeutic strategy thermogenic studies evaluated in the short and long term (6 months) is to combine with caffeine or ephedrine. However, this combination is not currently available for the treatment of obesity.

1.5.3.4 Drugs approved for other indications

Bupropion

Bupropion (BUP) is an atypical antidepressant that has also been studied for its effects on weight loss (Gadde and Xiong 2007). This amino ketone compound does not inhibit Monoamino oxidase (MAO) and is believed to provide neuropharmacological effects through the weak inhibition of reuptake NA, DA and 5-HT (Gadde et al. 2001), however, the mechanism contributing to the loss of weight not known. BUP is currently approved for the treatment of depression, and as an adjunct therapy to help people quit smoking.

Topiramate

Promoting weight gain is the most worrying side effect of many of the new antipsychotic medications, mood stabilizers and anticonvulsants. Topiramate is an

anticonvulsant with stabilizing properties of mood. In several uncontrolled trials, treatment with this drug resulted in partial or complete reversal of weight gain induced by other psychotropic drugs. This effect led to the investigation of topiramate as the primary treatment for obesity (Gordon and Price 1999).

Zonisamide

Zonisamide is an anticonvulsant atypical inducing weight loss in patients treated with other antiepileptic agents. This observation led to a randomized controlled trial in the short term, which demonstrated a significant weight loss in patients with moderate obesity (Gadde et al. 2003) .

1.5.3.5 Combination of drugs

Contrave® (BUP + Naltrexone). Combination therapy has proven more effective in treating a variety of conditions, from hypertension to heart disease and infectious disease. That is why the combination therapy implemented as a possible effective treatment in the fight against obesity. As already mentioned above the BUP is a reuptake inhibitor of NA and DA. Naltrexone is an opioid receptor antagonist that is ineffective by itself to induce significant weight loss (Greenway et al. 2009).

The combination of fenfluramine with phentermine, a sympathomimetic agent known as fen-pen was introduced in 1992 as an effective treatment for chronic obesity that was apparently free of serious side effects (Weintraub 1992). Although the focus of "fen-phen" was valuable to show that the drug long term is necessary to effectively treat obesity, problems with these agents were evident in 1996. In this year a group of hypertension cases primary pulmonary were related to the use of fenfluramine derivatives in Western Europe (Deitel 1997).

Empatic® (BUP + zonisamide). This combination of an antidepressant (BUP) and an anticonvulsant (zonisamide) caused a modest weight loss in obese people (Valentino et al. 2010).

Qnexa® (topiramate + phentermine). Phentermine is an amphetamine derivative that has anorectic effects and still is prescribed (even after failure of fenfluramine with fen-phen) in the USA for short-term treatment of obesity.

Topiramate is an antiepileptic drug with multiple mechanisms of action. These two drugs were combined to increase the efficacy and safety by reducing the individual daily dosages observed a weight loss of between 7 and 14%.

1.6 The brain as a target for the development of anti-obesity drugs

The classical idea of a central regulation of food intake and body weight has led to the concept that energy homeostasis results from the interaction between the central nervous system (CNS) and peripheral organs directly involved in body weight regulation such as the gastrointestinal tract and adipose tissue.

Electrical lesioning and stimulation studies in the hypothalamus have identified several nuclei that are important in the regulation of feeding behavior. Electrolytic destruction of the ventromedial hypothalamus increases ingestive behavior (by causing frequent meals consumption) and induces obesity (1983; Hoebel and Teitelbaum 1966; Thomas and Mayer 1968). In addition, lesions of the hypothalamic paraventricular nucleus (PVN) and the arcuate nucleus (ARC), which are highly interconnected nuclei, also result in hyperphagia and obesity (Choi and Dallman 1999; Leibowitz 1978). Moreover, electrical stimulation studies of the lateral hypothalamus (LH) activated a motor program that elicits eating and hoarding even when sated (Herberg and Blundell 1967), emphasizing the role of this nucleus in meal initiation and hunger signaling. Overall, these data implicate the hypothalamus in (the initiation of) ingestive behavior. In addition to the homeostatic control of feeding, which involves the hypothalamus, animals – including humans – also eat because palatable food is rewarding. It is hypothesized that this is because of the hedonic value of food (Woods and Seeley 2000).

Food reward and the anticipation of meals implicate the NAc – a brain structure that is important in reward processing – in the control of food intake (Hernandez and Hoebel 1988; Mendoza et al. 2005). The NAc is connected to both cortical and limbic brain regions (Powell and Leman 1976), and is implicated in motivation to eat (Kelley et al. 2005). NAc shell DA neurons have long been implicated in mediating reward behavior and the motivational aspects of feeding behavior (Palmiter 2007). The drugs that modulate (either stimulating or inhibiting)

receptors on NAc shell DA neurons, can affect the dynamics of DA signaling in the NAc, these could influence the subjective reward value of food and hence the motivation to eat.

1.7 Amphetamine like Appetite suppressants

Novel therapeutic strategies are being designed to treat obesity by acting on the main pathways communicating with the brain. The role of the brain in appetite regulation and body weight control is well known (Lopez et al. 2004). Thus, drugs targeting the CNS might represent the most promising obesity therapy. The first compound introduced as an appetite suppressant was amphetamine, which was addictive and had euphoric side effects (Drevets et al. 2001). To reduce these side effects, amphetamine-derived appetite suppressants (Fig. 3) such as **Diethylpropion (DEP), Phentermine (PHEN) and d-Norpseudoephedrine (NORP**- known as **cathine** an optical isomer of phenylpropanolamine designed to increase their appetite suppressant effect with reduced stimulant properties) were produced, and this is the most commonly prescribed agent (Bray 2000).

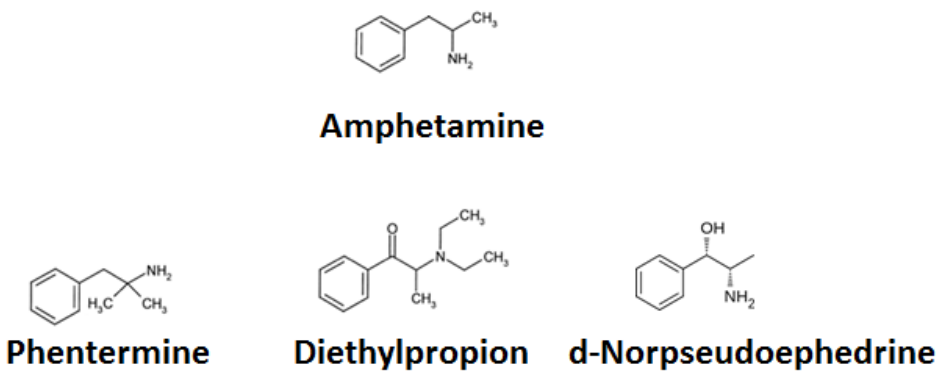

Figure 3. Amphetamine like appetite suppressants with their chemical structure.

Phenylpropanolamine (PPA), also known as the stereoisomers norephedrine and norpseudoephedrine, is a psychoactive drug of the phenethylamine and amphetamine chemical classes, which is used as a stimulant, decongestant, and anorectic agent. Phenylpropanolamine acts as an alpha-adrenergic receptor and beta-adrenergic receptor agonist as well as a DA (D1) receptor partial agonist (Flavahan 2005). There are four optical isomers of PPA: dextro- and levo- nor

ephedrine, and dextro- and levo- nor pseudoephedrine. D-norpseudoephedrine is also known as cathine, and occurs naturally in the plant Catha edulis ("Khat"). D-norpseudoephedrine, that is structurally an intermediate between (-)-cathinone and (+)-amphetamine. Kalix *et al* demonstrates that the khat alkaloid also has amphetamine-like effects at the cellular level (Kalix 1990).

1.7.1 Pharmacology

The noradrenergic sympathomimetic drugs (Table 1) are grouped together because they can increase blood pressure and in part act like the neurotransmitter NA. Drugs in this group work by a variety of mechanisms, including the release of NA from synaptic granules (DEP and PHEN), the blockade of NA re-uptake (mazindol), or direct action on adrenoceptors (phenylpropanolamine). All of these drugs are absorbed orally and reach peak blood concentrations within a short time. The half-life in blood is short for all except and its metabolites, which have a long half-life. Liver metabolism inactivates a large fraction of these drugs before excretion. Food intake is suppressed either by delaying the onset of a meal or by producing early satiety. Mazindol have been shown to increase thermogenesis in experimental animals, but the clinical data are contradictory.

1.7.2 Side-effects

Side effects also occur due to sympathetic stimulation and gastro-intestinal irritation. These side-effects may cause some individuals to stop taking the drug, but are never serious or dangerous. Drug interactions may occur with monoamine oxidase inhibitors and to a clinically unimportant extent, with antihypertensive drugs. The most common side effects of these appetite suppressants are restlessness, insomnia, dry mouth, constipation, and increased blood pressure and heart rate.

The anorectic drugs have a very definite part to play in the treatment of obesity, mainly for those individuals who have altered their eating habits but have come to a plateau of weight, which they find difficult to reduce. The drugs are best to given in a long-acting form and can be safely be continued as long as weight loss persists, if the clinician exercises careful supervision. Dexamphetamine, phenmetrazine and benzphetamine should rarely be used because of the danger of

addiction, and chlorphentermine is potentially hazardous for long-term use. PHEN emerges as the drug of first choice (monotherapy), as combined with fenfluramine has a tendency to cause depression and has a higher incidence (valvular insufficiency) of side effects. DEP and Mazindol appear to be useful as alternative drugs.

Table 1. Appetite suppressants drugs used to treat obesity.

Drug group	FDA approval	Approved duration of treatment	DEA schedule	Trade names	Dosage form (mg)	Administration
Noradrenergic drugs						
Norepinephrine releasers						
Diethylpropion	Yes	Few weeks	IV	Tenuate®	25	25 mg three times daily
Approved for short-term use				Tenuate®	75	75 mg once daily
				Dospan®		
Phentermine	Yes	Few weeks	IV	Standard	37.5	37.5 mg in a.m.
Approved for short term use				Adipex-P®	30	30 mg day 2 hours after breakfast
				Duromine®	37.5	37.5 mg/day 9 a.m.
				Fastin®	30	30 mg/day 2 hours after breakfast
				Obenix®	30	30 mg/day 2 hours after breakfast
				Oby-Cap®	30	30 mg/day 2 hours after breakfast
				Oby-Trim®		
				Zantryl®		
				Slow release		
				Ionamin®	15, 30	15 mg/day before breakfast (30 mg for less-responsive patients)
Norepinephrine re-uptake inhibitor						
Mazindol	Yes	Few weeks	IV	Sanorex®	1.2	Initial dose; 1 mg once a day Maximum dose: 1 mg three times a day with meals
Approved for short-term use				Mazanor®	1	Initial dose: 1 mg once a day Maximum dose: 1 mg three times a day with meals
Noradrenergic agonist						
Phenylpropanolamine	Yes	–	–	Dexatrim®	25, 75	25 mg three times daily
Approved for short-term use				Accutrim®		

1.7.3 Efficacy

The efficacy of an appetite suppressant drug can be established by showing that, in double-blind randomized clinical trials, it produces a significantly greater weight loss than the placebo (Ryan et al. 2009) and that the weight loss is more than 5% below base-line weight. Clinical trials of noradrenergic drugs performed before 1975 were generally short term because it was widely believed that short-term treatment would cure obesity (Gonzalez et al. 2007). Now increasing focuses on longer-term trials lasting over 24 weeks with an adequate control group (Bray 1993).

A 36-week trial comparing the continuous administration of PHEN with intermittent drug and placebo (Munro et al. 1968) Both continuous and intermittent PHEN therapy produced more weight loss than did the placebo. In the drug-free periods, weight loss slowed the intermittently treated patients, only for then patients lose weight more rapidly when the drug was reinstated. A small trial with

diethylpropion showed a greater weight loss than with placebo. PHEN and DEP are Schedule IV intravenous drugs a regulatory classification indicating the potential for abuse, although the potential appears to be very low. Until now PHEN and DEP are only approved (by FDA) for a few weeks, which is widely interpreted as being up to 12 weeks. Weight loss with PHEN and DEP persists for the duration of treatment, suggesting that tolerance does not develop to these drugs. If tolerance does develop, the drugs would be expected to lose their effectiveness or patients would require increased amounts of drug to maintain their weight loss. This does not seem to occur.

Clinical studies on PPA (Greenway 1992) 1439 subjects took active medication and 1086 placebo. At the end of the studies, which were up to 12 weeks in length and performed before 1985, subjects on phenylpropanolamine lost about 0.27 kg per week more than did subjects on placebo. There is only one controlled trial for phenylpropanolamine that lasted 20 weeks. In this double-blind placebo-controlled trial, 101 subjects were treated with placebo or phenylpropanolamine for 6 weeks with an optional double-blind extension to week 20 (Schteingart 1992). At 6 weeks, the phenylpropanolamine-treated group had lost 2.4 kg (0.43 kg per week) compared to wire 1.1 kg (0.18 kg per week) for the placebo group. In the optional extension, 24 subjects on phenylpropanolamine lost 5.1 kg (6.5%) compared with 12 subjects treated with placebo, who lost 0.4 kg per week (0.5%) of their initial body weight.

1.8 Appetite suppressants: Nucleus accumbens vs. Dopamine

Anorectic drugs may act mainly on the satiety center in the brain to produce anorexia. However, these drugs also have various peripheral metabolic effects involving fat and carbohydrate metabolism, but many of these may be secondary to loss of weight. Most of the drugs are related either directly or indirectly to amphetamine and in addition act by increasing physical activity in general. Anorectic drugs tend to lose their effect after some months (tachyphylaxis), and part of this reduction in effect may be due to chemical alterations produced by the drugs in the brain. All the drugs, amphetamine like analogs have stimulant effect on the central

nervous system in some individuals, resulting in restlessness and nervousness, irritability and insomnia. Euphoria occasionally occurs with DEP, PHEN and chlorphentermine, but to a much lesser extent.

These amphetamine like appetite suppressants drugs acting through the CNS affect neurotransmitters with anorectics or appetite suppressant actions. These treatments have targeted three monoamine receptor systems namely, the noradrenergic, dopaminergic, and serotonergic systems. Carr and its collaborators (Carr and White 1986) examined the contributions of DA terminal regions to these effects in rats by microinjecting amphetamine directly into one of six discrete brain sites (medial frontal cortex, nucleus accumbens, anteromedial caudate nucleus, ventrolateral caudate nucleus, amygdala, or the region surrounding the area postrema) and observing the effects of the injections on eating or drinking. The rats were mildly deprived of either food or water and following microinjection of either amphetamine or saline, were given access to food or water. Injections of amphetamine into either the NAc or amygdala caused both anorexia and adipsia but no effects were observed from the other sites. In addition, produced changes in activity level from several of the injection sites (more pronounced with NAc microinjection) and there was increase in the behaviors associated with stereotypy also (Carr and White 1987).

However, amphetamine infusion into the NAc shell did not increase food intake, and in fact reduced it at the highest dose tested, interestingly showed a trend to increase their intake at lower doses which showed a bidirectional effect for AMPH for ingestive behavior. In addition, dose dependent increased appetitive behaviors towards food (See Fig. 4) was also noticed. Also, intra-NAc shell infusions of DA receptor antagonists, regardless of the subtype selectivity of the antagonists or the NAc into which they are injected, dramatically reduces the motor output associated with a hungry state without reducing (or in some cases, actually increasing) food intake (Baldo et al. 2002; Barbano and Cador 2006). Taken together, these studies suggest that the effects of striatal (NAc shell) dopaminergic manipulations on food intake and appetitive behaviors. Although striatal dopamine is involved in behavioral

activation and increasing appetitive behaviors toward food, it does not appear to directly modulate the ingestive behavior (Kelley et al. 2005).

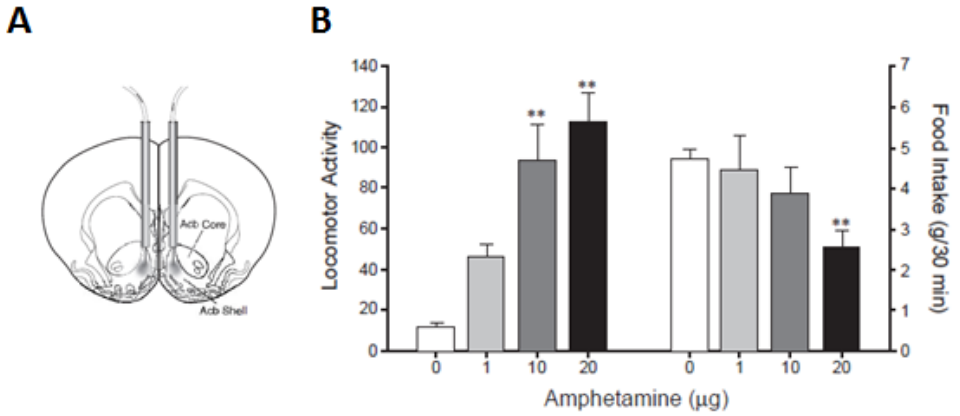

Figure 4. The effects of amphetamine on food intake and appetitive behaviors. **(A)** Coronal section of rats demonstrating the infusion of AMPH into the NAc shell using cannula. **(B)** Infusion of AMPH into the NAc shell specifically did not increase food intake, and in fact reduced it at the highest dose and increase the locomotor activity (appetite behaviors), compared to vehicle infusions. It seems AMPH in NAc shell does not appear to directly modulate ingestive behavior or food intake. (Taken from, Kelley et al. (2005))

Amphetamine exerts its effects primarily by stimulating activity at catecholaminergic synapses. It stimulates the release of DA and NA and blocks their deactivation by reuptake (Fuxe et al. 1970). Several lines of evidence have suggested that it is primarily amphetamine's stimulation of dopaminergic activity that results in the observed changes in open field activity. These studies have tended to divide amphetamine's effects dichotomously into effects observed at lower vs. higher doses of the drug. Increased locomotion has been associated with lower doses and stereotypy with high doses. Both amphetamine-induced locomotor activity and stereotypy were antagonized by inhibition of DA synthesis (Randrup and Munkvad 1966; Weissman et al. 1966) but inhibition of NA synthesis (from DA) or noradrenergic antagonists were ineffective (Randrup et al. 1963). Also, it has been reported that amphetamine, methylamphetamine, propylhexedrine, phentermine, mephentermine and p-chloroamphetamine probably produce their stimulant action by a release of catecholamines from neuronal extra granular pools (Samanin et al. 1975). Chlorphentermine and diethylpropion probably produce stimulation by a release of catecholamines from neuronal granular pools (Offermeier and Potgieter

1972). Further, it has been shown that AMPH induced both the locomotor and stereotyped responses are dependent on the functional integrity of the nigrostriatal dopamine pathway (Creese and Iversen 1975).

DEP, has been examined previously for effects on DA levels (Santamaria and Arias 2010; Yu et al. 2000). The effects of DEP on DA efflux in adolescent rat accumbens broadly agree with the both in vitro assessing pre-loaded titrated DA efflux and in vivo using micro-dialysis. Yu et al., (2000) showed that diethylpropion and its primary metabolite ethcathinone had inhibitory concentration (IC) 50s of approximately 15 and 1 µM respectively in an assay for dopamine uptake. DEP was tested for stimulant effects *in vitro*, through assessing their abilities to increase basal and electrically evoked dopamine efflux in rat accumbens brain slices, and found that DA efflux produced by DEP was significantly increased at 30 and 100 µM. Although, DEP per se is not a direct substrate releasers at the DA transporter (DAT) (Opacka-Juffry et al. 2014), thus DEP needs to be metabolized in order to release DA in the NAc. PHEN administration to intact, awake rats increases brain DA release in striatum without affecting 5-HT. This effect also shared with d-amphetamine, but not with dexfenfluramine (Balcioglu and Wurtman 1998). Both a1-adrenergic and D1-dopaminergic neurotransmissions are involved in phenylpropanolamine-mediated food suppression in mice (Cheng and Kuo 2003).

The major problem with the results of these numerous studies in obesity is that there are significant discrepancies between them, not just as to which areas of the brain are activated and which not, but even as to the direction of the activation. There are a number of reasons behind this variability. These amphetamine like appetite suppressant drugs, introduced into the market after FDA approval in the 1960's, was thought to act, like its analog d-amphetamine, as a ¨sympathomimetic agent with mild stimulant properties¨ probably mediated by the catecholamines (might also involve dopamine- Samanin, Bernasconi et al. 1975), i.e. noradrenaline with little abuse potential.(Samanin and Garattini 1993). However, no direct evidence is available yet. It is therefore crucial that electrophysiological studies are always accompanied with direct behavioral testing techniques and the overall pattern of

these complimentary methodologies are needed to uncover the electrophysiological correlates for this class of appetite suppressants.

2. JUSTIFICATION

Obesity is considered a chronic medical disease state (Kushner 2014). Dieting and exercising to lose weight require substantial effort, willpower and persistence, making weight-reducing drugs a coadjuvant in the treatment of obesity. Consequently, more and more strategies focusing on obesity treatment are being explored. Over the past six decades, research has unraveled neural circuits that are implicated in the regulation of eating. The entry of food into the stomach and intestines and the delivery of nutrients to the liver generate neural signals to the brain, via the vagal nerve, that have a role in the termination of a meal. However, to develop effective pharmacological therapies against obesity, we must understand the neuronal mechanism of these appetite suppressants. Only then will it be possible to ensure efficacy, safety, and sustainable weight loss.

DEP, PHEN and NORP (amphetamine congeners) have been used largely for the short term treatment of weight loss over 55 years (Hampp et al. 2013). However, these drugs are known as noradrenergic drugs the mechanisms by which these pro-drugs produce their anorexic effect are complex and not well known. They modulate the concentrations of 5-HT, NA and DA to evoke responses in various cortical and subcortical areas involved in eating. Despite, several differences from a neurochemical perspective, of particular interest is DA since in rodents all these drugs (either directly or throughout its metabolites) cause a release of DA at the NAc shell (Balcioglu and Wurtman 1998; Santamaria and Arias 2010), a brain region receives DA inputs and it is involved in eating and motor behavior (Kelley et al. 2005; Palmiter 2007). Additionally, in rodents the parent compound (AMPH) in the NAc shell was shown to stimulate locomotion and its appetite/ingestive behavior (Kelley et al. 2005). In order to shed light about their central mechanism of action in the brain reward region, we characterized the behavioral and neuronal responses evoked by the short term treatment of DEP, PHEN, and NORP.

3. HYPOTHESIS

Appetite suppressants modulate the spiking activity of NAc shell neurons, and they act via activation of D1R and D2R DA receptors.

4. GENERAL OBJECTIVE

To determine whether DEP, PHEN and NORP mediate their effects via activation of NAc shell DA receptors.

4.1 Specific objectives

1) To determine the dose response behavioral (food intake and stimulant effects like locomotion and stereotypy-head weavings) effects induced by these appetite suppressants.

2) To understand the neuronal responses evoked by these appetite suppressants drugs in NAc shell, by using single-unit recording of spikes and local field potentials (LFP´s) in freely moving rats.

3) To determine the role of DA receptors (D1R and D2R) in modulating the behavioral and neuronal responses induced by these appetite suppressant drugs.

5. MATERIALS AND METHODS

5.1 Animals

A total of 168 male Sprague-Dawley rats ~250-300 g were used for all experiments. Animals were housed individually and had *ad libitum* access (unless otherwise mentioned) to food and water except when they were removed from their home cages for testing in an operant box for multichannel recordings (see below) or in an open field arena for locomotion and stereotypy studies. Room temperature was maintained at (21 ± 1 °C) on a 12/12 hour light-dark cycle (0600-1800 h). All procedures were approved by the CINVESTAV institutional animal care and use committee (CICUAL).

5.2 Drugs and Chemicals

The appetite suppressants drugs: diethylpropion hydrochloride (DEP), d-norpseudoephedrine hydrochloride (NORP) and phentermine hydrochloride (PHEN) were kindly donated by the pharmaceutical company Productos Medix® (Mexico). R (+) – SCH-23390 hydrochloride (SCH) and S (-) – Raclopride (+) tartrate salt (RAC) were obtained from Sigma Aldrich. Unless otherwise mentioned, these compounds were dissolved in physiological saline (0.9% NaCl) and administered intraperitoneally (i.p.) for the dose-response effect behavioral effects of appetite suppressant drugs on weight-loss, food suppresion and locomotion. Lithium chloride (LiCl) and sodium saccharin was obtained from Sigma Aldrich and was dissolved in water for conditioned taste aversion (CTA) experiments. For all electrophysiological experiments drugs were delivered intragastrically (i.g.) (DEP and PHEN) and i.p. (NORP) using a catheter (see below), in a volume of 1 mL/Kg.

5.3 Dose dependent behavioral effects of appetite suppressants

5.3.1 Dose-response effect of appetite suppressants upon weight-loss and food intake

To determine whether appetite suppressant drugs (DEP, PHEN and NORP) affects body weight and food intake in a dose-response manner, 70 lean male rats were assigned into different groups. The dose dependent effect of DEP was studied using different doses namely 0, 1, 5, 10, 80 mg/Kg (n=5/group), regarding PHEN 0,

1, 3, 10, 30 mg/Kg (n=5/group) and NORP (n=4/group) with 0, 10, 20, 40 and 80 mg/Kg were used. Animals were placed in individual home cages where they were provided with 100 g per day of standard rat chow (Purina Mexico) and *ad libitum* water. After seven days (to obtain a stable baseline- data not shown), rats received a daily i.p. injection of one of the assigned treatments for seven consecutive days (days 1-7). These behavioral experiments were carried out between either 2 or 4 h (i.e., 14:00 -18:00 h) before the commencement of rat's active (lights-off) phase. Their body weight and 24 h food intake was measured daily 20 min before receiving an i.p. injection of the corresponding treatment.

5.3.2 Dose-response effect of appetite suppressants upon locomotor-activity and stereotypy in open field arena

In same group of rats as described above, we evaluated a dose-dependent effects on locomotion and stereotypy (head weavings) over seven days of repeated i.p. injection of amphetamine like appetite suppressant drugs. Unless otherwise mentioned all these locomotor effects were measured using Ethovision XT10 (Noldus Information Technology, Netherlands). Of maximum 6 individual open field arena (40L X 40W X 30H cm) were serially placed together in two rows (3X2) and recorded with a CCD camera (IDS camera, Germany) with specialized software (uEye Cockpit) to grab the activity (top view) with the resolution of 15 fps (fps-Frames per second). At first, three-color points (Red, Green and Blue) were glued to the animal and selected as targets for the nose, head and body, respectively. Later, assigned groups of animals were injected with their respective treatments and placed individually in the open field for 90 min. As mentioned above all the experiment were conducted between 14:00-18:00 h in two phases (14:00-16:00 and 16:00-18:00 h). After completion of each session the grabbed video files were analyzed (only videos where locomotion could be clearly decoded were included in the analysis) using *Ethovision XT10* three body (nose, center mass and tail base) point detection method. The animals center body mass was tracked as "x" and "y" coordinates to calculate the distance moved during first the 90 min after receiving their respective treatment. For stereotypy measurements, we focused on head weavings since they were most readily measurable and quantifiable. We note that

other stereotypic behaviors e.g., licking the walls and wood gnawing behavior, were also observed (mainly in the higher doses). We did not quantify other types of stereotypy since for the dose primarily used in most experiments, head weavings were the most prominent stereotypy. For head weavings the nose targets were tracked using the same tracking method described above. The lateral head movements of the animal after injection was calculated as turn angle and expressed as stereotypy as the occurrence of a turn angle more than 45°. In both cases, homemade MATLAB scripts calculated the locomotion (in cm/90min) and stereotypy (counts/90min).

5.4 Neuronal response of appetite suppressants in NAc shell.

5.4.1 Implantation of intragastric/ intraperitoneal catheter for drug infusion

For the implantation of the intragastric catheter was made using the protocol adopted from Lukas and Moreton (1979); Ueno et al. (2012). During multichannel recordings (see below), animals were either intragastrically (i.g.) or intraperitoneally (i.p) infused with appetite suppressant. The two basic reason for choosing intragastric infusions of appetite suppressants, mimic how humans take these compounds (orally) and not to disturb the animals during electrophysiological recordings by handling. Regarding the anorectic effect for NORP, our pilot studies found to be effective only for intraperitoneal administration, so we decided to do electrophysiological studies for NORP using intraperitoneal catheter.

Briefly, the hair on the abdomen and dorsal neck areas was clipped and cleaned. To expose the stomach, a midline incision of 2-3 cm was made in the abdomen. With a 30-gauge needle we punctured the fundus and inserted 1 cm of the catheter into it (14 cm length and 0.76 mm diameter of silastic laboratory tubing; Dow Corning, USA, with one sterile rubber band braces glued at 1 cm from the tip). Then, we joined the catheter's rubber band and the rats' stomach using a non-absorbable silk suture (USP 6-0, ATRAMAT Mexico). For intraperitoneal catheterization, ventral peritoneal incision was made and catheter was inserted (1 cm) and fixed using nylon stitches. Later, we passed the opposite end of the catheter (for both i.g. and i.p.) subcutaneously until it exited the dorsal neck incision. Finally,

the peritoneal cavity was carefully stitched together using chromic Catgut, whereas the abdominal and dorsal incision was stitched together using a silk suture. After the animals were permitted to recover, at least one-week from surgery before starting the treatment, they were habituated to the operant box for 2 days. In each experiment, a infusion tube (30 cm length) was connected to the catheter and to a syringe that was manually activated outside of the behavioral box. The catheter patency was always confirmed at the end of all experiments before perfusion of the animal.

5.4.2 Implantation of multi-channel electrode in NAc shell

After i.g. / i.p. cathertization, surgical procedures was continued for electrode implantation in the NAc followed methods described previously (Tellez et al. 2012). Briefly, animals (300-350 g) were anesthetized using an intraperitoneal injection of cocktailed Ketamine (100 mg/Kg) and Xylazine (8 mg/Kg). A movable 4 x 4 microwire array comprised of formvar-coated tungsten wires (35 µm diameter) was unilaterally implanted in the NAc shell (centered from Bregma at coordinates: AP = 1.4, L = ±1, and DV = 7.5 mm). One stainless steel screw that was soldered to a silver wire (50 µm) at the surface of cerebellum served as ground. Finally the electrode was anchored to the skull using dental acrylic supported by two more screws screwed in the skull bone.

5.4.3 Multichannel recording for spikes and local field potentials (LFP´s) from the NAc shell in freely moving rats

All recordings began one-week after the post-surgery recovery period. They were performed in an operant behavioral box that was enclosed in a ventilated and sound-attenuating cubicle equipped with a camera. Unless otherwise mentioned each recording session lasted 3 h and was divided into three 1 h epochs: baseline, infusion of saline and infusion of an appetite suppressant with or without a DA antagonist. Action potentials and LFPs were recorded using a Multichannel Acquisition Processor (Plexon, Dallas, TX). Only single neurons with action potentials with a signal-to-noise ratio larger than 3:1 were analyzed. Action potentials were identified online by means of voltage-time threshold windows and a three principal components contour template algorithm (Gutierrez et al. 2010). Spikes

were sorted using off-line sorter software (Plexon Dallas, TX) and the stability of waveform shape across the 3 h recording session was confirmed by plotting the average shape during baseline, saline and appetite suppressant epochs.

LFPs recorded from the NAc shell, were amplified 1000X, filtered 0.7–300 Hz and digitized at 1 kHz using a digital acquisition card (National Instruments, Austin, TX). For each LFP, the power spectral density (PSD) was estimated with the Welch's method using a 2 s Hanning window with 50% overlap. Channels with exceptionally large noise levels, based on visual inspection, were excluded from analyses. From the remaining channels when an artifact event occurred (e.g., saturations and abrupt changes in voltage) the segments 100 ms before to 300 ms after the event were eliminated. The PSD of each LFP was averaged across all channels to generate a single PSD plot per experiment. We also report the average PSDs at delta (1-4 Hz), theta (6-9 Hz) and beta (15-30 Hz) bands (Halje et al. 2012). For comparison across experiments, PSDs were normalized to their z-score by subtracting their means and dividing them by the standard deviation (std's) of the baseline period. To allow time for the drugs to achieve a steady pharmacological state, the LFPs 5 min before and 10 min after the start of each epoch were not analyzed.

5.4.4 Measurement of locomotion during multichannel recordings from the NAc shell

During multichannel recordings, the position of the rat's center of mass was tracked by using *Ethovision XT10* (see above for detailed methodology) and reported as cm/min. In addition, only webcam videos where locomotion could be clearly decoded were included in the analysis. Onset of locomotion was computed by using a cumsum statistic (Gutierrez et al. 2006) to obtain the first bin after drug infusion where locomotion significantly increased above locomotion in the saline epoch.

5.4.5 Neuronal effects of appetite suppressant drugs on the NAc shell activity

For these experiments we used 11 animals with electrode arrays implanted in their NAc shell (DEP20 n=4, PHEN20 n=4, and NORP20 n=3). Drug infusions were made via i.g. / i.p. infusions via catheters. The animal's body weight and 24 h food intake were measured daily across 7 days 20 min before starting a recording

session. It is important to note that in our pilot study, despite having food available rats practically do not eat or drink anything during the initial 2-3 h after drug administration, where these drugs exert their maximal locomotor effects. Given this information, we decided not to have food available during the multichannel recording sessions since rats will simply ignore it and also because the delivery of food pellets would interfere with our ability to accurately decode locomotion from the videos.

5.5 Data analyses

All analyses were performed using matlab toolboxes and homemade custom scripts.

5.5.1 Active and Inactive neurons: after DEP, PHEN and NORP administration

Neuronal firing modulations such as those shown in Figures 10, 19A and D were identified using a Kruskal-Wallis test for $\alpha < 0.05$. Firing rates were pooled across baseline and saline epochs since differences between them were not significantly different (Table 2). Moreover, after DEP, PHEN and NORP infusions, because rats remained >80% of the time in the active awake state (Table 5). We compared the firing rates of the baseline-saline period, during the quiet wake times (computed from the hypnogram, more details see below) against the firing rates during active awake (from the time interval 2:10 to 3 h) after the infusion of the appetite suppressant drugs.

5.5.2 Putative neuron-type classification

Neurons were classified into putative cell-types according to four features: firing rate, coefficient of variation 2 (CV2), valley-to-amplitude ratio (VAR), and valley-to-peak width (V-P width) (see Tellez et. al. 2012). The firing rate was calculated as the number of spikes divided by the duration of baseline and/or saline periods (that were not significantly different; see Table 2). CV2 was calculated for each adjacent pair of inter-spike intervals (ISIs), and the average CV2_ISI was used in the assignment of neuron type. The two-ISI coefficient of variation was computed as $CV2\_ISI = |2(ISI2 - ISI1)/(ISI2 + ISI1)|$ (Holt et al. 1996). The VAR was calculated as the absolute value of the first valley in the waveform divided by the difference between its minimum value and the following maximum (Fig. 12A and (Yarom and

Cohen 2011)). For computation of the V-P width (the time between the minimum value and the following maximum was calculated), see black waveform in Figure 12A, black waveform shape (pFSI). For each neuron recorded, these four features were computed and classified into 4 clusters by using a fuzzy cluster algorithm, and visualized by using principal component analysis (PCA) as seed followed by the fuzzy Sammon's mapping plot as described in the Matlab Fuzzy Clustering & Data Analysis Toolbox.

5.5.3 Hypnograms: the LFP's brain state map

For hypnograms (Fig. 13B; right panel), behavioral states were assigned using information obtained from the LFPs as outlined in (Gervasoni et al. 2004; Tellez et al. 2012). In brief, after elimination of segments with amplitude saturation, a sliding window Fourier-transform was applied to each LFP signal to calculate two spectral amplitude ratios (0.5–20/0.5–55 Hz and 0.5– 4.5/0.5–9 Hz for ratio 1 and 2, respectively). PCA was then applied to these ratios obtained from all LFP channels, and the PCs were used as the overall ratios measure. These measures obtained for each second of data were further smoothed with a Hanning window (20 s length). Finally, the two PCs of the spectral ratios were plotted against each other to construct a two-dimensional (2-D) state space (Fig. 13B) where the density of points reflecting the relative abundance of the different brain states. REM sleep was not included in this analysis because the percentage of time the animals spent in REM was < 2% (unpublished observation). The final two-dimensional brain state map was selected and validated after visual inspection of animals behavior in the videotaped with three behavioral states (quiet wake (qW), slow wave sleep (SWS) and active wake (aW) (Tellez et al. 2012).

5.6 IntraNAc shell infusions of DA antagonists:

5.6.1 Implantation of cannula

IntraNAc shell infusions of DA antagonists were performed to directly determine their effects on weight-loss, eating and locomotion. For the bilateral intraNAc shell infusions two holes were drilled at: AP +1.4 mm; L ± 1 mm relative from Bregma. The tips of stainless steel guide cannula aimed at the NAc shell were

bilaterally inserted at 6.5 mm DV from Bregma. Two screws served as anchors in the skull bone and the whole assembly was cemented to the skull with dental acrylic. A stylus was inserted into the cannula (to prevent clogging) and was removed before each infusion. Rats were allowed to recover from surgery for at least seven days.

5.6.2 IntraNAc shell injection

Microinfusions were given via a 30-gauge stainless steel injector 1.0 mm larger than the tip of the guide cannula, connected via a Teflon tubing to a 10-µL glass micro syringe (Hamilton 80366) that was attached to a microinfusion pump (KD scientific- KDS200 series). A total volume of 0.5 µL (0.33 µL/min) per hemisphere was infused daily across 7 days of treatment. The injector was left into the guide cannula one additional minute to allow complete effusion (Gutierrez et al. 2003).

5.6.3 Effect of D1 (SCH) and D2 (RAC) antagonists infused in the NAc shell upon appetite suppressant drugs effect on weight loss, food intake, locomotion and stereotypy

To investigate whether D1 and/or D2 receptors in the NAc shell mediates some (or all) of the pharmacological effects induced by appetite suppressants. For DEP, we used 18 animals randomly sorted into 6 groups (n = 3) namely, Sal+Sal, SCH+Sal, RAC+Sal, Sal+DEP20, SCH+DEP20 and RAC+DPE20. After 2 days of habituation (data not shown), each animal was placed one by one in the individual open field for 90 min and their locomotor activity was analyzed using an RV2 video processor (RV2, TDT systems, Alachua, USA -digital videotaped and real-time tracking). The RV2 video analyzer was placed in an open field arena (40L X 40W X 30H cm) containing a CCD camera (top view) with specialized software (VGAC Color Video Camera; RV map, TDT systems) to track the animal's position in "x" and "y" coordinates. Further, homemade MATLAB scripts was used to calculate the locomotion (in cm). After the initial 20 min baseline period, animals were briefly removed from the open field and were injected with 2.5 µg/0.5 µL of either SCH or RAC antagonists directly in the NAc shell (Baldo et al. 2002; van den Boss et al. 1988). After 30 minutes (Fig. 16A), all rats received the corresponding systemic infusion of either saline or DEP20 via the i.g. catheter. These behavioral experiments

were carried out between 07:00 -19:00 h (light phase). Their body weight and chow food intake was measured daily just before placement in the open field.

Similar to DEP, PHEN and NORP was also evaluated for the role of DA receptors in the NAc shell for it anorectics effects with a slight modifications. Similarly classified groups as in DEP experiment, here with 27 naïve animals (n=3) using same control groups (Sal+Sal, SCH+Sal and RAC+Sal) for both PHEN20 and NORP20. Instead of individual arena here, we used multiple arena and tracked using *Ethovision XT10*. After 45 min (for DEP just 25 min) of baseline animals were injected with either D1 or D2 antagonist (2.5 µg/0.5 µL) in NAc shell by taking out from the box and again at 60 min animals received their respective treatments (either saline or PHEN20/NORP20) as intraperitoneal injections. All this experiments were carried out 2h before the commencement of the rat´s active (dark) phase. Their body weight and chow food intake was measured daily just before placement in the open field (Figs. 16 B and C).

5.7 The effect of intragastric D1 (SCH23390) and D2 (raclopride) antagonists on DEP20's effect on weight loss, food intake, locomotion and stereotypy

To understand the participation of D1Rs and D2Rs on DEP's behavioral effects, we tested and compared four groups of each comprised of three animals: saline, DEP20, DEP20+RAC0.5 and DEP20+SCH1.5 where the numbers are in mg/Kg. Briefly, rats were habituated to the open field arena for two days (data not shown). Then each group received a daily i.g. infusion for seven days where their locomotion and stereotypy were measured. We initially used this method because i.g. infusions of appetite suppressants mimic how humans take these compounds. As seen above, we also infused these antagonists directly into the NAc shell.

To determine whether DEP-induced neuronal modulation could be reversed by i.g. infusion DA receptor antagonists, we first infused DEP20 followed 30 min later by one of the two antagonists (Fig. 18). The antagonist concentrations were selected based on previous studies showing that RAC at a systemic dose of 0.5 mg/Kg antagonized the locomotion effects induced by amphetamine or methamphetamine (Broening et al. 2005; Janhunen et al. 2013; Wright et al. 2013). Likewise, we initially

tested using a high dose of SCH (3 mg/Kg), since this dose is used to prevent death from an overdose of amphetamine (Derlet et al. 1990). However, over a seven days test period animals generally did not tolerate this dose, and thus we could record NAc shell activity in only one animal as seen in Figure 19. Consequently, we used SCH at 1.5 mg/Kg for the seven-day treatment period.

As a control we also tested if these DA antagonists by themselves produce any behavioral effects. This was accomplished using two additional control groups with each one receiving either only RAC0.5 or SCH1.5. Specifically, rats were introduced on the open field arena during 30 min as baseline (BL), followed by an i.g. injection of saline at 30 min and at 60 min (instead of DEP) they received an injection of either RAC0.5 or SCH1.5 (see Fig. 18C). Finally, at 90 min, they received a second infusion of saline.

5.8 Intragastric NAc shell D1R and D2R antagonists' modulation of NAc shell activity

Finally we wonder to evaluate the effects of DA receptor antagonists upon modulating DEP20's effects on NAc shell activity, we implanted 10 animals with multichannel electrodes in their NAc shell (DEP20+Rac0.5 n=2 rats; 81 neurons, and DEP20+SCH1.5 n=2 rats; 112 neurons, DEP20+SCH3, n=1; 34 neurons, RAC0.5 n=2; 93 neurons, and SCH n=3; 114 neurons). Experiments were performed in an operant chamber and each epoch was 30 min long. In order to increase the statistical power we pooled together the baseline and saline epochs (that were not significantly different, Figs. 19A & D) and compared it against the firing rate on the DEP20 and antagonist epochs using a Kruskal-Wallis test. The normalized z-score peristimulus time histogram (PSTH) of significantly modulated neurons was obtained as were the normalized PSDs at beta, theta and delta bands (Figs. 19B and E*).* We tracked the animal's locomotion (Figs. 19C and F), based on the center mass point method described above. Finally, to compare the global effect of DEP20 under chronic treatment of the above mentioned D1R or D2R antagonists, the population firing rate of all recorded neurons (modulated or not by DEP) were plotted aligning its response to time 0 min as the moment of DEP20 infusion, and

normalizing their firing rate using the 30 min saline period as a baseline (Figs. 20A and B).

5.9 Behavioral effect of DEP20 as unconditioned aversive stimuli

Twelve naïve rats were randomly divided into two groups: LiCl (n=5) and DEP20 (n=7), following the protocol described in (Gutierrez et al. 2003). Briefly, animals were water deprived for 23.75 h and for three days, they had a daily period of 15-min access to water (Fig. 22A, W1-W3). On day 4 (ACQ, acquisition day), animals were allowed to drink a novel saccharin solution (0.1% w/v) for 15 min that served as conditioned stimuli, and 15 min later rats received a single intraperitoneal injection of either 0.4 M LiCl – 7.5 mL/kg- or DEP20 - 1 mL/Kg - that served as unconditioned aversive stimuli. On days 5 and 6, animals were given only water to allow recovery from gastric malaise. On the TEST day (day 7) rats were presented with 15 min (17:30-17:45 h) of 0.1% w/v saccharin followed by other 15 min of water (17:47–18:02 h). Following the TEST session, rats received three more extinction days (EXT1-3). Intake (mL) was measured at 0.5 mL resolution and displayed in Figure 22A.

5.10 Modulation of NAc shell's LFP oscillations induced by a gastric malaise agent (LiCl)

To understand whether DEP20-induced LFP's oscillations are related to any aversive-malaise brain state, we evaluated whether a prototypical gastric malaise agent, LiCl, was able to alter LFP's oscillations in the same way as DEP20 did (Figure 13). We used 2 animals with a microarray of electrodes implanted in the NAc shell and with an i.g. catheter (see above). A total of 5 recording sessions were successfully performed (we let at least 2 days of rest between each recording session to allow recovery from the sickness induced by previous injection of LiCl). To reduce animal distress we did not inject LiCl more than 3 times per animal. Each recording session contained four, 1 h, epochs: Baseline, Saline, LiCl (0.4 M) and DEP20, respectively. LFP's oscillations and locomotion were analyzed and displayed in Figures 22 B-D. From these experiments, we could only record a few single unit neurons (n = 12). Given this small sample size and because none of

these neurons were significantly modulated by LiCl (data not shown) we could not make any conclusions from these data.

5.11 Histology

At the end of the experiments rats were i.p. injected with pentobarbital sodium (150 mg/Kg) and perfused with PBS, followed by 4% paraformaldehyde (PFH). Their brains were removed and placed in 10% sucrose/PFH (vol/vol) solution for 24 h followed by sequential increases in sucrose/PFH concentration until 30% at 72 h. To establish the placement of the electrodes brains were sectioned (50 µm) and stained with cresyl-violet.

5.12 Statistical analyses

Unless otherwise mentioned data is presented as mean ± *SEM*. Statistical differences between groups were assessed by a one-way ANOVA or repeated-measures ANOVA (RM ANOVA), followed by Fisher's post-hoc analysis.

6. Results

6.1 Behavioral effects of appetite suppressants

6.1.1 Effect of appetite suppressants upon weight-loss and food suppression

Diethylpropion (DEP)

Initially we went to determine an optimal concentration of these appetite suppressants to induce weight-loss in rodents. Figure 5A (top panel) shows a graph of the change in body weight across 7 days of daily intraperitoneal injections of: saline, 1, 5, 10 and 80 mg/Kg of DEP (hereafter noted as DEP1, 5, 10 and 80, respectively). With saline injections (control) it is seen that the animals progressively gained weight until the trial ended on day 7. Interestingly, DEP1 showed a faster trend (than saline) to gain their bodyweight. In contrast, increasing the DEP concentration caused a dose-dependent increase in weight loss. Specifically, for DEP5 initial decrease in weight gain (until day 3) later whose effect decreased across the treatment period and remains similar to saline. Whereas DEP10 reduced the normal weight gain but DEP80 showed a transient decrease in weight that was maintained across the entire treatment period. The overall mean (± *SEM*) change in body weight across the seven treatment days was 5.9 g ± 1.1, 8.9 g ± 2.4, 5.5 g ± 1.8, 0.3 g ± 2.5, and -20.1 g ± 3.7 for saline, DEP1, 5, 10 and 80, respectively (main effect of dose: RM ANOVA $F_{(4, 20)} = 22.3$, $P < 0.0001$; days: $F_{(4, 6)} = 5.4$, $P < 0.0001$; and dose x days interaction: $F_{(24, 120)} = 6.0$, $P < 0.0001$). *Post-hoc* comparisons, relative to saline, indicated that DEP80 induced a significant weight loss ($P < 0.0001$). In summary, DEP induces a dose-dependent decrease in body weight.

In the same group of animals we also measured their food intake over 24 h (Fig. 5A, bottom panel). Although over 7 day treatment the saline-injected animals exhibited a relatively constant food intake. At a lower dose DEP1 increased their food intake relative to saline across all the treatment days. In contrast the animals under treatment with the other doses initially decreased food intake in a dose-dependent manner. DEP5 decreased their food intake just on day 1 later it follows similar to saline. DEP10 showed a marked decreased in the food intake and for DEP80 after the large decrease in food intake on day 1, the animals increased their

intake but still remain below from that of the other treatments. These results show that over the treatment period the animals developed tolerance to the anorectic effects of DEP, but they consistently consumed 5-10% less chow food than saline group. Over the 7 day treatment period, the percentage change of food intake from the day before treatment started was 2.4 ± 2.9% (mean ± *SEM*), 13.2 ± 6.2%, 1.8 ± 3.3%, -13.9 ± 3.4% and -35.9 ± 4.9 %, for saline, DEP1, 5, 10, and 80 respectively (main effect of dose: RM ANOVA; $F_{(4, 20)} = 19.5$, $P < 0.0001$; and days: $F_{(4, 6)} = 8.7$, $P < 0.0001$ but no significant interaction between dose and days: $F_{(24, 120)} = 1.30$, $P = 0.1769$). Although the animals under treatment initially decreased food intake in a dose-dependent manner with DEP80 showing the greatest reduction (*post-hoc*, $P <0.0001$), followed by DEP10 ($P = 0.015$) but DEP5 was not significant ($P = 0.93$, n.s). Interestingly, DEP1 showed a trend to ate more food than saline but not significant ($P = 0.094$, n.s.).

Overall our results suggest that similar to amphetamine, DEP can also induced a bidirectional effect over weight-loss and food intake. At lower doses increase food intake and weight gain, as upon increasing the dose DEP exhibits a dose dependent weight loss and food suppression effects in rats.

Phentermine (PHEN)

In addition to DEP, we tested another commonly used appetite suppressant, PHEN, in a new cohort of animals to determine their effects on weight loss and food intake. Like DEP, PHEN (Fig. 5B, top panel) exhibits a dose dependent weight-loss. As expected 7 days treatment with saline increase their bodyweight, whereas PHEN treatment decreased their weight gain in a dose dependent manner. PHEN1 gained weight faster than control (Saline) group like DEP1, whereas PHEN3 fails to show an anorectic effect. However, a reduction in weight gain was noticed at PHEN10 and at a dose of 30 mg/Kg PHEN induce a stronger weight loss across all the treatment days. Specifically, across seven days of treatment the change in body weight (mean ± *SEM*) was: saline (6.4 ± 1.3 g), PHEN1 (8.3 ± 1.5 g), PHEN3 (5.7 ± 1.4 g), PHEN10 (0.9 ± 2.3 g) and PHEN30 (-16.0 ± 2.7 g), respectively. In summary RM ANOVA showed a significant effect between groups (main effect of treatment; $F_{(4, 20)} = 26.4$,

$P < 0.0001$), across 7 days ($F_{(4, 6)} = 13.3$, $P < 0.0001$) and significant interaction between treatment and days ($F_{(24, 120)} = 12.5$, $P < 0.0001$). Further *post-hoc* analysis revealed that only PHEN30 significantly reduced the bodyweight ($P < 0.0001$) when compared with control.

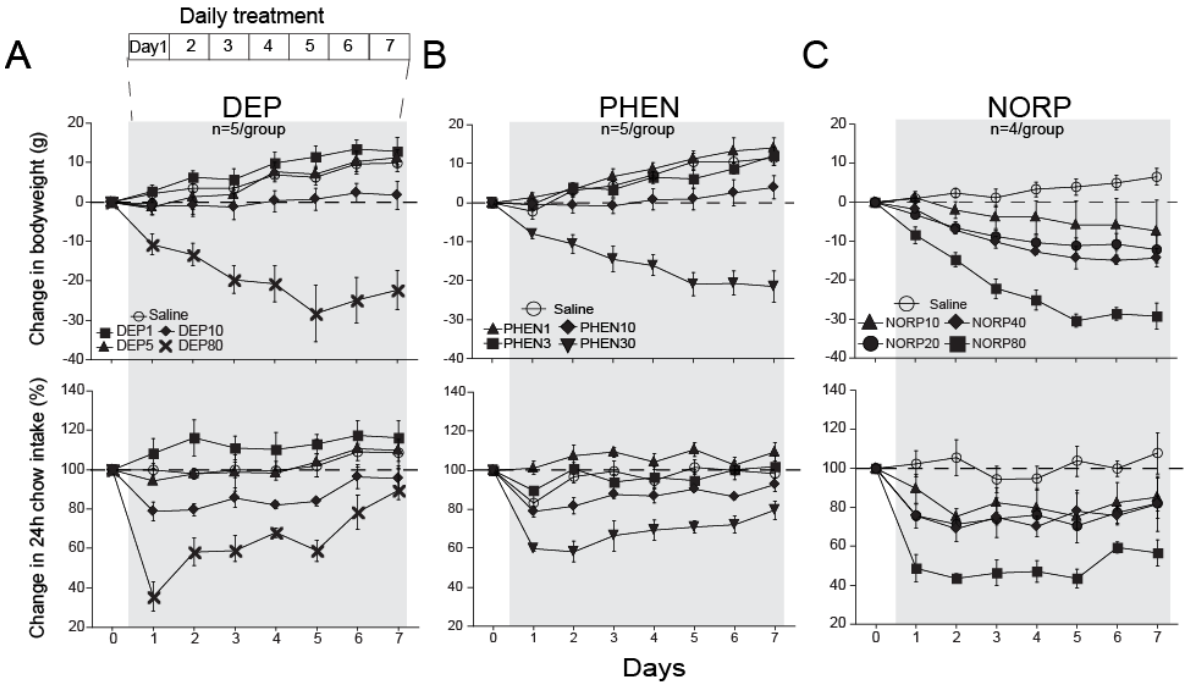

Figure 5. Effects of appetite suppressant drugs on weight loss and food intake. (A) The change in body weight of control rats daily injected intraperitoneally with saline (○) for 7 days compared with rats injected with diethylpropion (DEP) at 1 (■), 5 (▲), 10 (⧫) and 80 (🗙) mg/kg. Gray shading depicts treatment periods. The change in body weight and food intake measured 20 min before each injection. The horizontal dotted line represents no weight change. The DEP-induced change in food intake for the same subjects shown in top panel. **(B)** Top: graph showing the change in weight over a 7-day i.p. treatment (gray shading) with saline and different doses of phentermine (PHEN-1, 3, 10 and 30 mg/Kg). Bottom: for the same animals, the change in 24-h food intake. **(C)** The change in bodyweight (Top panel) and 24 h chow intake (bottom panel) over a 7 day i.p. treatment with control (saline) and different doses of d-norpseudoephedrine (NORP-10, 20, 40 and 80 mg/Kg). Symbols represent means ± SE.

Figure 5B, bottom panel displays the percentage change in food intake for the same animals as above. Percentage change of food intake from the day before treatment started was -3.7 ± 3.8% (mean ± *SEM*), 6.4 ± 2.7%, -3.2 ± 2.7%, -13.7 ± 1.3% and -31.9 ± 4.0 %, for saline, PHEN1, 3, 10, and 30 respectively. RM ANOVA showed a significant effect between groups (main effect of dose; $F_{(4, 20)} = 22.3$, $P <$

0.0001), across 7 days ($F_{(4, 6)}$ = 11.3, P < 0.0001), but no significant interaction between dose and days ($F_{(24, 120)}$ = 1.5, P = 0.06, n.s). Further *post-hoc* analysis significantly revealed the bidirectional effect of PHEN on feeding behavior. PHEN1 significantly (P = 0.03) increased their feeding whereas PHEN 10 (P = 0.03) and 30 (P < 0.0001) significantly showed a food suppression effect over 7 days of treatment when compared with control.

In summary these result conclude that PHEN induces a dose dependent appetite suppressant effects in rats and also it was more potent than DEP in food suppression and weight-loss effects.

Norpseudoephedrine (NORP)

To determine how effective NORP20 to induce weight loss and food suppression effects in rats. Figure 5C, top panel shows the change in body weight during short term treatment with 4 different doses of NORP (10, 20, 40 and 80 mg/Kg) and a control (Saline). Throughout the 7 days, we observed that control groups continued gaining body weight. In contrast, and relative to saline treated groups, the NORP groups showed a dose dependent reduction in bodyweight gain. The overall mean (± *SEM*) change in body weight across the seven treatment days was 3.2 g ± 1.6, -7.3 g ± 7.8, -9.1 g ± 1.8, -12.3 g ± 2.3, and -29.3 g ± 3.4 for saline, NORP10, 20, 40 and 80, respectively [(RMANOVA- $F_{(4, 15)}$ = 13.1, P = 0.0001), and significant effect for days ($F_{(4, 6)}$ = 25.02, P < 0.0001) with a significant interaction ($F_{(24, 90)}$ = 4.4, P < 0.0001)]. Furthermore, a *post hoc* analysis uncovered that NORP group showed a faster and greater weight loss in a dose dependent manner (P < 0.0001, P = 0.003 and P = 0.006 for NORP80, 40 and 20 respectively). Although NORP10 induces weight loss but the post-hoc analysis found to be not significant relative to saline (P = 0.08, n.s). Interestingly, NORP20 and NORP40 was found to be equally effective to induce weight loss (P = 0.62, n.s). Whereas NORP80 induced a greater weight-loss, this difference of course was due to the faster and continuous induction of weight-loss without any tolerance effects. Our results indicate that NORP is an effective anorectic drug with less tolerance (tachyphylaxis), unlike DEP and PHEN.

In the same animals shown above, we also measured the percentage change in 24 h food intake relative to last day before the treatment (Fig. 5C, Bottom Panel). Again, group treated with 7 saline injections ate constant food throughout the experiment. Despite that as noted above, they continuously gained body weight. As expected NORP groups ate less food when compared with their respective saline controls. The difference between the groups was statistically significant ($F_{(4, 15)}$ = 9.2, $P < 0.0007$) with a significant effect across days ($F_{(4, 6)}$ = 3.4, P = 0.005). The *post hoc* analysis indicates that despite that the NORP10 was less effective to induce weight-loss (Fig. 5C, Top panel), but it exhibited a significant food suppression throughout the 7 days of treatment (P = 0.04). However the food suppression effect was also significant for NORP80, 40 and 20 ($P < 0.0001$, P = 0.009 and P = 0.01 respectively) relative to saline. Hence our results demonstrated that the appetite suppressant drug NORP is effective to induce weight loss and decrease food intake with lesser tachyphylaxis.

6.1.2 Effective Dose 50 (ED_{50}) for appetite suppressants

Additionally to the dose response studies, we also evaluated the effective dose response for those appetite suppressants to induce weight loss in rodents. Sigmoidal curve was plotted (Fig. 6) to know the effective dose 50 (ED_{50}) to induce the bodyweight loss with respect to the control as 0 percentage and the higher dose as 100 percentage. The forms in the curve represents the doses of the each drug are maintained same as indicated above in Figure 5.

For DEP and PHEN as shown above (Fig 5A and B top panels, for DEP and PHEN respectively) the low dose (DEP1, filled squares and PHEN1, filled triangles) was ineffective to induce weight loss perhaps it increase the weight gain so that we see a negative weight loss for DEP1 and PHEN1 in the sigmoidal curve. The weight-loss for DEP and PHEN was not induced until DEP5 (See filled triangle of Fig. 5A) and PHEN3 (See filled squares of Fig 5B). The effective dose to induce 25 percent weight loss (ED_{25}) was found to be 11 mg/kg for DEP, 10 mg/Kg for PHEN and 6 mg/Kg for NORP. In comparison with these three appetite suppressants, PHEN found to be potent to induce weight-loss with effective dose (ED_{50}) was 15 mg/Kg

whereas DEP at 17 mg/Kg and NORP at 20 mg/Kg. Even though PHEN was potent their therapeutic index is smaller to induce weight loss. ED_{75} for PHEN, DEP and NORP was found to be 20, 29 and 46 mg/Kg respectively. Moreover, LD_{50} for DEP, PHEN and NORP was found to be 140, 50 and 120 mg/Kg.

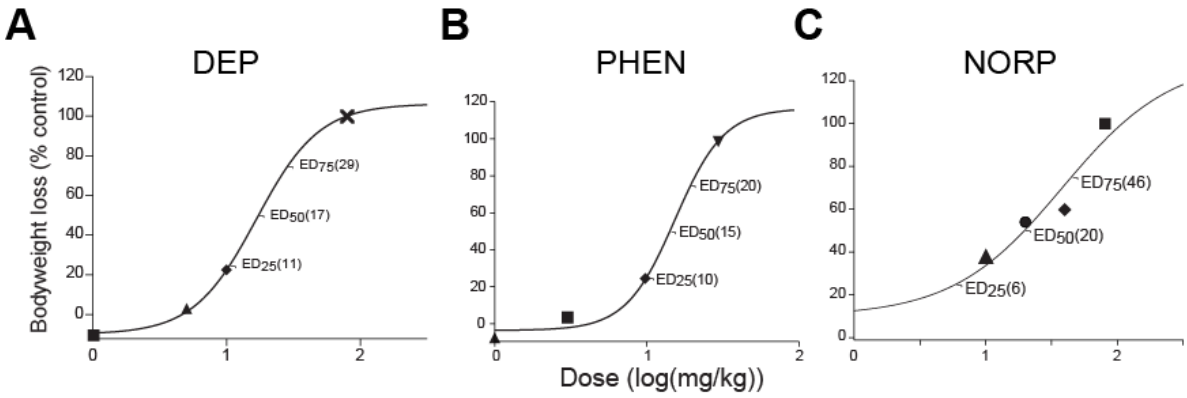

Figure 6. Sigmoidal dose-response curve of appetite suppressants. Effective dose range (ED_{25}, $_{50}$ and $_{75}$) to induce bodyweight loss with respect to control was plotted using a sigmoidal curve **(A)** DEP, **(B)** PHEN and **(C)** NORP respectively. The forms in the curve represents the doses of the each drug as indicated in Figure 5.

6.1.3 Effect of appetite suppressants on locomotion and stereotypy

DEP

Previous studies with rats reported that acute administration of DEP alters locomotion and produces stereotypy (Reimer et al. 1995). Here we asked how a dose-dependent chronic treatment affects locomotion and stereotypic behavior (head weavings). In the same cohort of animals as in Figure 5A (n=25), we evaluated the effects of seven-day i.p. injections of different doses of DEP on their locomotion (Fig. 7A). Saline injection did not display marked changes in locomotion with less than 500 cm/90min (normal exploration of the open field). However continuous 7 days of DEP treatment increase locomotion in a dose dependent manner with the maximal effect (17215±389 cm/90min) at an intermediate dose (DEP10) and impairs its locomotor activity (12085±321 cm/90 min) on higher doses (DEP80). DEP5 exhibits less locomotor effects even though significant than control. Seven days of different dose of DEP treatment showed a significant main effect of dose (RM ANOVA; $F_{(4, 18)} = 3.9$, $P = 0.02$; and significant effect of days: $F_{(4, 6)} = 4.2$, $P = 0.0009$, with significant interaction $F_{(24, 108)} = 1.7$, $P = 0.03$). A *post-hoc* analysis

showed that DEP10 (P = 0.002), DEP80 (P = 0.01) and DEP5 (P = 0.02) induced a significant locomotion than saline. Moreover, DEP1 also increased their activity but not significant in comparison with saline (P = 0.34, n.s.).

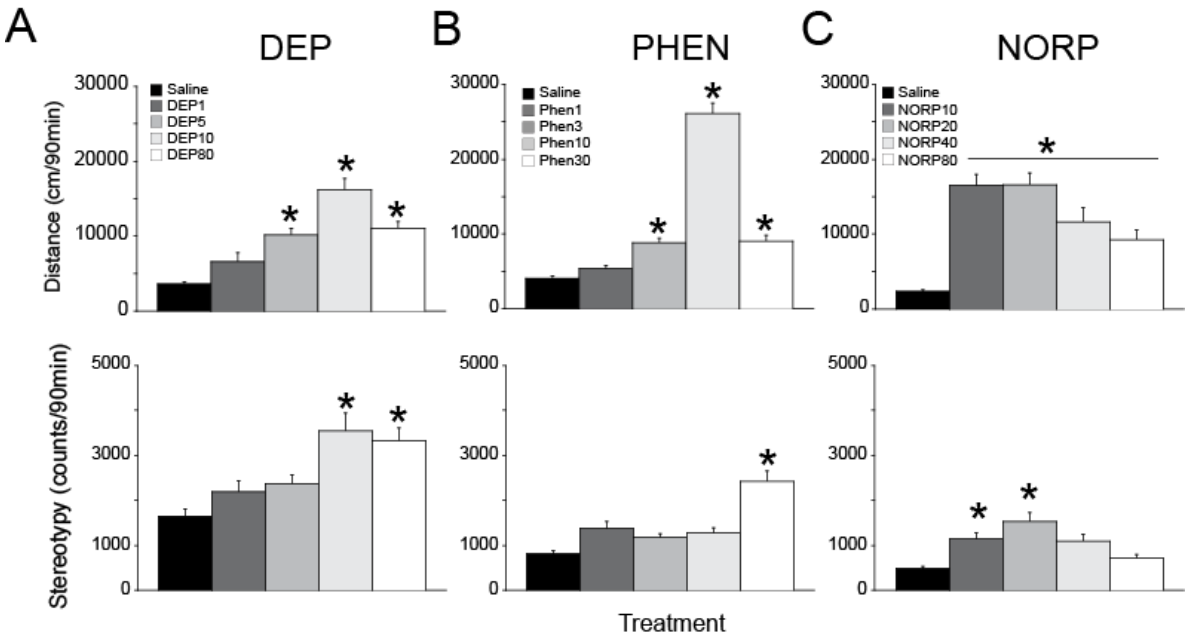

Figure 7. Effects of appetite suppressants on locomotion, and stereotypic head movements. (A) Effect on locomotion (distance moved during 90 min) after repeated injections (7 days of treatment) of different doses of DEP and saline measured in an open-field arena. Bottom: quantification of head weavings (stereotypy) caused by DEP. **(B)** Top: graph showing the locomotor activity over 90 min period for 7-days of repeated i.p. injection of with saline and different doses of phentermine (PHEN-1, 3, 10 and 30 mg/Kg). Bottom: for the same animals, the head weavings were measured. **(C)** The distance moved (Top panel) and stereotypy (bottom panel) over a 7 day i.p. treatment with control (saline) and different doses of d-norpseudoephedrine (NORP-10, 20, 40 and 80 mg/Kg). Symbols represent means ± SE. *P < 0.05 compared against their respective saline treatment.

With respect to DEP-induced changes in stereotypy (Fig. 7A, bottom panel), saline injections induced a normal exploration behaviors with the lateral head movements. However, 7 days of DEP treatment increases the stereotypic head movements (RM ANOVA; significant main effect of doses: $F_{(4, 18)}$ = 3.10, P = 0.04, significant effect of days $F_{(4, 6)}$ = 2.4, P =0.03 and no significant interaction dose x days $F_{(24, 108)}$ = 0.44, P = 0.98). Briefly, DEP10 (3,509± 431 counts/90min) and DEP80 (3,304± 233) produced a stronger stereotypic behavior and found to be significant (P = 0.01 and P = 0.04 respectively) against saline but almost similar between them (P = 0.13, n.s). Whereas the others (DEP1 and DEP5) doses did not

markedly change their head weavings so the responses were not statistically different (P = 0.3 and P = 0.24 respectively). These data show that seven days of treatment with DEP induces stereotypy in a dose dependent manner.

PHEN

We then measured how PHEN (Fig. 7B) affect locomotion (upper panel) and stereotypy (lower panel) for 90 min after drug administration. Saline group remained consistently low whereas locomotion significantly increased to PHEN3, PHEN10 and PHEN30 (RM ANOVA: main effect, $F_{(4, 20)}$ = 53.1, P < 0.0001). Effect across 7 days of treatment was not significant ($F_{(4, 6)}$ = 0.19, P = 0.98) and no significant interaction between [group X days] ($F_{(24, 120)}$ = 1.37, P = 0.14). PHEN10 induced a greater locomotion (26,462 ± 388 cm/90min, P < 0.0001) than PHEN30 (9,115 ± 291, (P = 0.009) and PHEN3 (8,951± 233, P = 0.01) and also significantly increased when compared with saline treated group (4,503±158). In summary like DEP, PHEN treatment induced a dose dependent locomotor activity (arousal) but gradually decreased (locomotor impairment) with higher dose.

For stereotypy measurements (Fig. 7B, bottom panel), saline group also exhibits a normal exploratory stereotypic head movements, whereas all doses of PHEN treatment induces head weavings but only PHEN30 induced a greater effect. PHEN30 induced almost twice the number of head weavings (2,403 ± 207 counts/90 min) as PHEN1 (1,417 ± 275), PHEN3 (1,062 ±185) and PHEN10 (1,262 ±185). RM ANOVA showed a significant difference across treatment groups ($F_{(4, 20)}$ = 35.4, P = 0.004), effect across days: $F_{(3, 6)}$ = 3.3, P = 0.042 and interaction between treatment and days: $F_{(24, 120)}$ = 4.4, P = 0.03). Furthermore, *post-hoc* analysis revealed that only PHEN30 significantly (P = 0.0007) induced stereotypy than control.

NORP

Like other appetite suppressant NORP also increases locomotion and gradually impairs its locomotion at higher doses. Significant increase in locomotion was observed with all the doses of NORP (RM ANOVA, $F_{(4, 11)}$ = 6.5, P = 0.006) with a significant effect across days ($F_{(4, 6)}$ = 4.5, P = 0.006) and interaction across treatment and days ($F_{(24, 66)}$ = 1.9, P = 0.01). Both NORP10 (17,254±454 cm/90 min,

P = 0.001) and 20 (17,326±394, *P* = 0.001) induced greater locomotion and not significant between them (*P* = 0.84, n.s) whereas on increasing the dose of NORP to 40 (11,326±441, *P* = 0.04) and 80 (9,526±402, *P* = 0.02) locomotion gradually decreased but remain significant than saline treated group.

NORP also induced stereotypic head weavings relative to their control group but less pronounced than DEP and PHEN. Similar to locomotor induction stereotypy also increased in a dose dependent manner until NORP20 later decreased over increasing the dose. RM ANOVA was significant across the treatment groups ($F_{(4, 11)} = 6.7$, $P = 0.03$) and significant across the 7 days of treatment ($F_{(4, 6)} = 2.4$, $P = 0.04$). Further *post-hoc* analysis elucidates that only NORP10 (*P* = 0.03) and NORP20 (*P* = 0.004) induced a stronger and significant stereotypy. Although NORP40 and 80 also induced stereotypy higher than saline but not significant. However NORP induced locomotion was similar to other amphetamine like appetite suppressant drugs but their induction of stereotypy was less pronounced which is consistent with the previous data (Kalix et al. 1990) that this specific (dextro-) stereoisomer is less effective to induce stereotypic behavior without reducing its potency over its pharmacological effects.

6.1.4 Comparison of appetite suppressant drugs

In order to understand the neuronal mechanism of these appetite suppressant drugs in the NAc shell for its appetite suppressant effect (although ED_{50} of these drugs are different) we titrated with different doses of those drugs to obtain a similar anorectic and appetite suppressant effect. Figure 8 plotted the behavioral effects induced by the three appetite suppressants, relative to saline infusions, all the three DEP20, PHEN20 and NORP20 caused a reduction in body weight. Specifically, across seven days of treatment the change in body weight (mean ± *SEM*) was: saline (8.1 ± 1.2 g), DEP20 (-8.9 ± 1.7 g), PHEN20 (-5.8 ± 0.7 g) and NORP20 (-9.1 ± 1.8 g), respectively. A RM ANOVA showed a significant effect between groups (main effect of treatment; $F_{(3, 14)} = 26.8$, $P < 0.0001$), across 7 days ($F_{(3, 6)} = 4.3$, $P = 0.0008$) and significant interaction between treatment and days ($F_{(18, 84)} = 5.8$, $P < 0.0001$). Furthermore, DEP20, PHEN20 and NORP20 also

decreased food intake: saline (1.2 ± 4.5%), DEP20 (-14.9 ± 6.0%), PHEN20 (-15.5 ± 3.3%) and NORP20 (-24.8 ± 2.7%) (RM ANOVA: $F_{(3, 14)} = 4.9$, $P = 0.02$; effect across days $F_{(3, 6)} = 18.7$, $P < 0.0001$; interaction between groups and days: $F_{(18, 84)} = 4.5$, $P < 0.0001$). In comparison to saline, NORP20 ($P = 0.002$), DEP20 ($P = 0.03$) and PHEN20 ($P = 0.02$) significantly reduced food intake (*post-hoc* test), which demonstrates that NORP20 exhibits less tachyphylaxis than DEP20 and PHEN20.

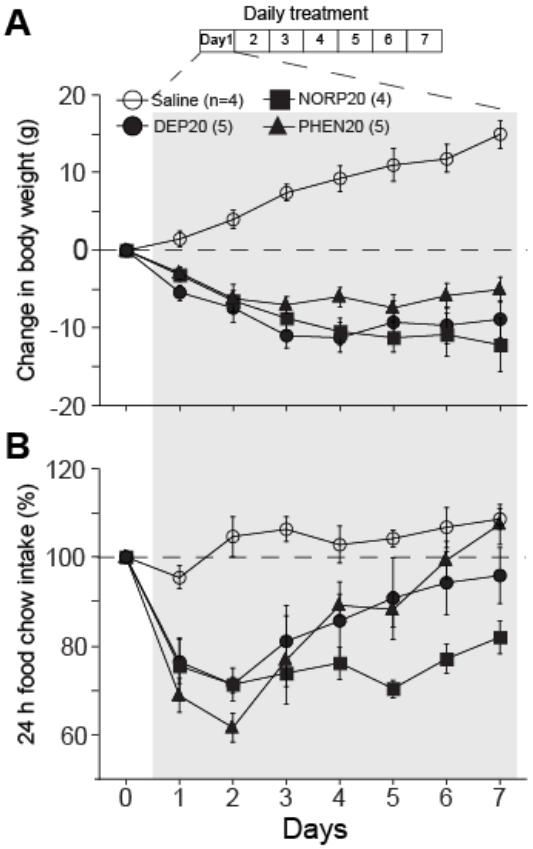

Figure 8. Comparison of appetite suppressants to induce weight-loss and food intake. (A) Graph showing the change in body weight over a 7-days (gray shading) of i.p. treatment with saline, DEP20, PHEN20 and NORP20. (B) The appetite suppressant drugs-induced change in food intake for the same subjects shown in top panel.

6.2 Electrophysiology

6.2.1 Appetite suppressant drugs strongly modulates NAc shell neuronal activity

To relate the behavioral studies to neural recordings we recorded the responses to the appetite suppressants with an specific and effective dose (DEP20, PHEN20 and NORP20) in the NAc shell, a brain region involved in reward, feeding

and motor activity (Brown et al. 2011; Kelley et al. 2005; Li et al. 2012). Unless otherwise stated, all electrophysiological recordings followed the same protocol. That is, after 1 h baseline period where the animals were free to move, they received a daily infusion of saline (at 1 h) and appetite suppressant drug at 2 h. Recordings were usually terminated after 3 h. A representative activated response (left panel) from a neuron and a representative inhibited response from a neuron (right panel) are illustrated as well with their waveform pattern across the DEP20 experimental session (Fig. 9).

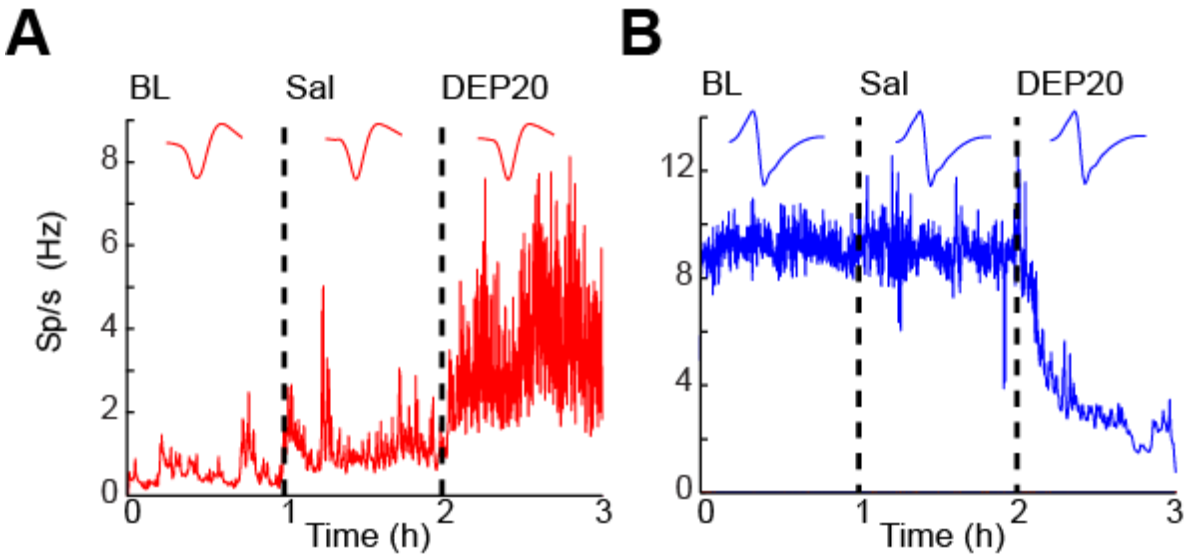

Figure 9. Intragastric infusions of DEP20 modulates nucleus accumbens (NAc) shell spiking activity. (**A**) Excitatory changes from a representative neuron with their waveform during baseline (BL), saline (Sal), and DEP20 infusion. (**B**) Inhibitory response of a representative neuron for the BL, Sal, and DEP20 epochs. Note that the firing rate dramatically increased (A) and decreased (B) after infusion of DEP20.

With respect to changes in firing rates, except for the abrupt increases (possibly the transitions between sleep and awake states) there is not a marked change in spiking activity from baseline to saline (see Fig. 9A, firing rates of Activated neuron: baseline = 0.57, saline= 1.1 Sp/s; inhibited neuron: baseline = 9.1, saline 8.9 Sp/s). In contrast, about 5 min after the infusion of DEP20 there is a marked change in inhibition and/or activation that lasted more than 1 h (Activated: 3.4 Sp/s after DEP20 and Inhibited: 3.1 Sp/s after DEP20; Fig. 9A). These changes are representative of the population responses (Table 2).

Table 2. Firing rates as a function of drug

		Firing rate (Sp/s) per 60 min epoch		
Drug	Type of response	Baseline	Saline	Drug
DEP20	Activated (n=14)	3.1 ± 0.01	3.1 ± 0.04	7.9 ± 0.18 *
	Inhibited (n=82)	5.7 ± 0.01	6.0 ± 0.03	2.0 ± 0.04 *
PHEN20	Activated (n=3)	2.8 ± 0.01	2.6 ± 0.02	6.2 ± 0.08 *
	Inhibited (n=38)	3.9 ± 0.01	3.8 ± 0.01	1.6 ± 0.02 *
NORP20	Activated (n=13)	2.2 ± 0.01	2.2 ± 0.02	4.3 ± 0.04 *
	Inhibited (n=18)	2.4 ± 0.01	2.4 ± 0.01	0.8 ± 0.01 *

Values are mean ± SEM. * P < 0.05, comparison across epochs.

Figure 10A shows a graph containing 131 neurons that were recorded before and after the infusion of DEP20. Relative to the not significantly different baseline and saline epochs (Table 2), two types of modulatory responses were observed: those whose activity decreased (inhibited by the appetite suppressant drug DEP20; blue dots (63% (82/131)) and those that increased (activated by the appetite suppressant drug DEP20; red dots 11% (14/131; $\chi^2_{(1)} = 51.9$, $P < 0.0001$). The normalized individual and population activity changes for 96 (82 inhibited and 14 activated) of the 131 neurons that significantly changed their firing rate in response to DEP20 are seen in Figure 10A (black traces). These data clearly show that NAc shell activity is strongly modulated by DEP20. The remaining 35 neurons (26%) were unaffected.

With other group of naïve animals we recorded the activity from their NAc shell during the baseline, saline, PHEN20 (Fig. 10B) or NOR20 (Fig. 10C) epochs. As with DEP20, both these compounds evoked both excitatory and inhibitory response in firing rate. Information about the average firing rate of activated and inhibited neurons by the three appetite suppressants are shown in Table 2.

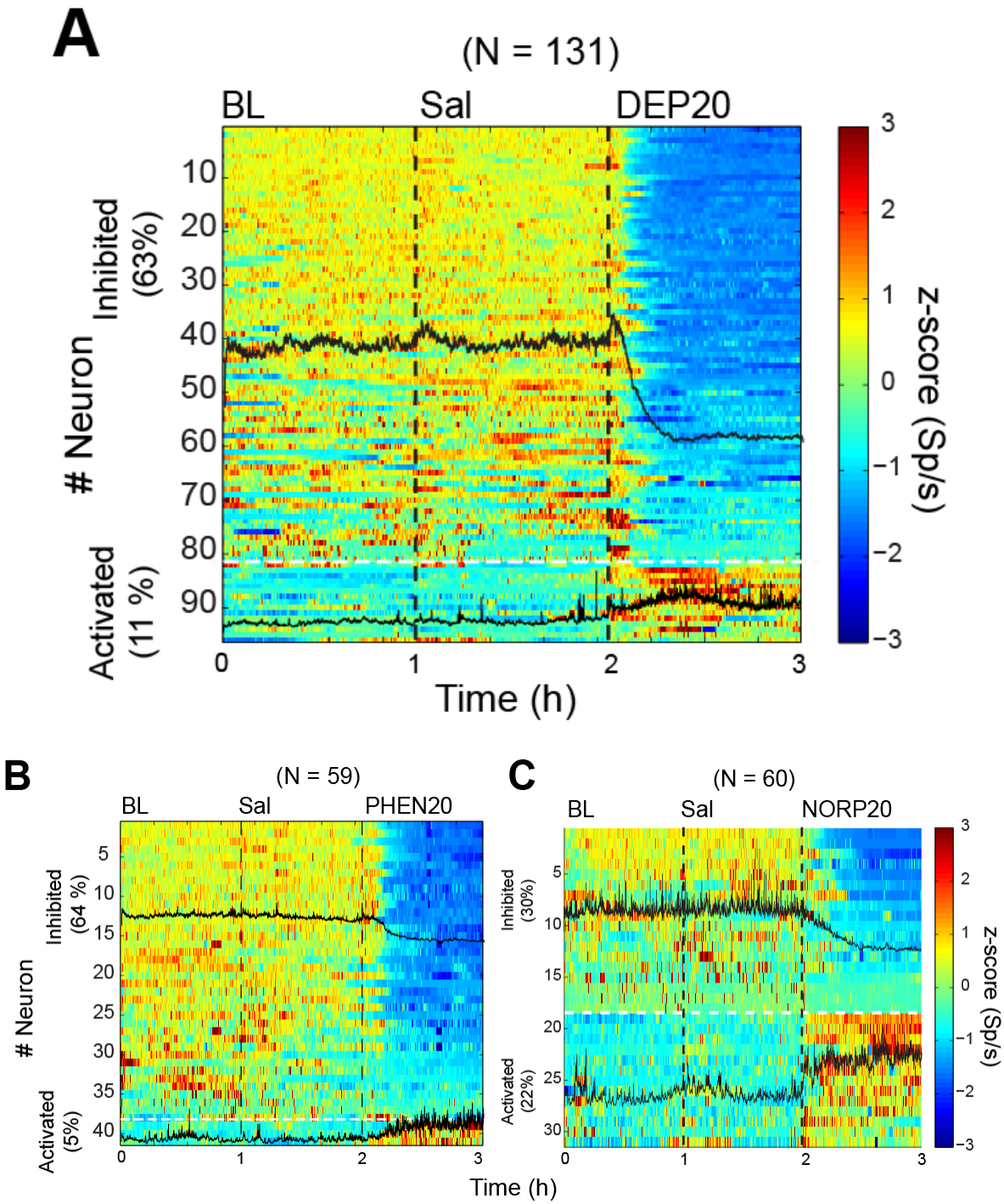

Figure 10. Administration of appetite suppressant drugs modulates nucleus accumbens (NAc) shell spiking activity. (**A**) Population responses of 96 (of 131) neurons recorded during the BL, Sal, and DEP20 epochs. The activity of each neuron is normalized to its z-score value and is plotted in a color-coded population peristimulus time histogram (PSTH) showing that 63% (n = 82; black overlap line indicates average population response) were inhibited by DEP20 and 11% (n = 14) were activated. The other neurons (26%) were unaffected. Each neuron was normalized to z-score values, color-coded PSTH showing the

number of NAc shell neurons overlaid with the black tracings are the mean PSTH of the inhibited and activated responses by (**B**) PHEN20 [Inhibited- 64% (38/59) and activated- 5 (3/59)] (**C**) NORP20 [Inhibited- 30% (18/60) and activated- 22% (13/60)]. The horizontal dotted white line indicates the division between inhibited and activated responses.

PHEN20 was most similar to DEP20 in that it produced 64% (38/59) inhibited responses and only 5% activated (3/59; $\chi^2_{(1)} = 23$, $P < 0.0001$) (compared with 63% ($P = 0.91$ n.s.) and 11% for DEP20 ($P = 0.24$ n.s.). The application of NORP20 although induced a greater number of inhibited (30%, 18/60) than activated (22%, 13/60) responses but not significant ($\chi^2_{(1)} = 0.64$, $P = 0.42$, n.s). The proportion of inhibited responses induced by NORP20 was significantly different than for both DEP20 and PHEN20 (over 7 days: $\chi^2_{(2)} = 6.7$ $P = 0.04$). Activated responses were also significantly different among drugs (NORP20 (22%), PHEN20 (5%) and DEP20 (11%); $\chi^2_{(2)} = 6.2$, $P = 0.04$).

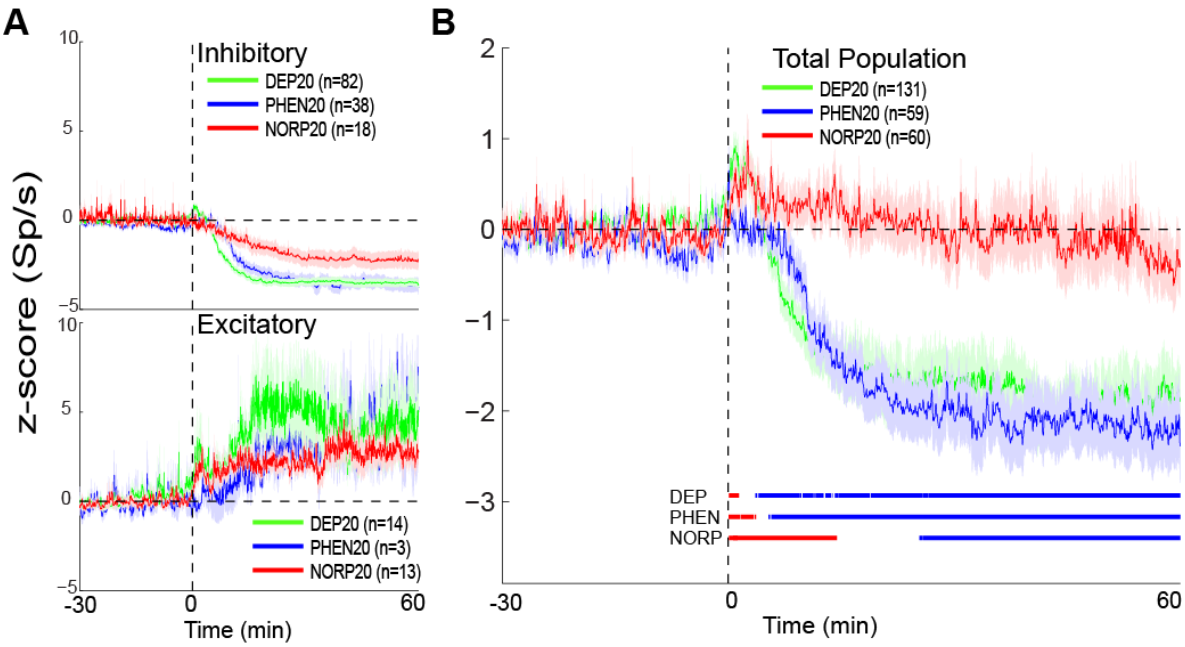

Figure 11. Appetite suppressants induces a stronger inhibition: activation response in the NAc shell spiking activity. (A) A plot of normalized (to its z-score) inhibitory (Top panel) and excitatory (bottom panel) population recorded during DEP20 (green traces), PHEN20 (blue traces) and NORP20 (red traces). **(B)** The normalized global population responses of all neurons recorded (n = 131) with DEP20 alone (green; aligned to DEP injection time = 0 min) and all neurons recorded with PHEN20 (59-in blue traces) and NORP20 (60-in red traces) respectively. The colored lines at bottom indicate the bins (1-min resolution) with significant increases (red) or decreases (blue) of population activity relative to saline firing rates (time interval: −30 to 0 min).

Note that the infusion of both DEP20 and PHEN20, induced a stronger inhibition but lesser by NORP20 in the NAc shell which remain until an hour (Fig. 11A top panel). On the other hand, induced activation which found to be little higher in magnitude (than inhibition) but just in few neurons [DEP20 (n=14), PHEN20 (3) and NORP20 (13)], however NORP20 activation (red traces, Fig.11A, bottom panel) also remains less strong than DEP20 and PHEN20 (green and blue). Although the magnitude of activation was stronger the total population activity over a 7 day treatment period for both DEP20 and PHEN20 produced a stronger inhibitory imbalance of NAc shell activity (Fig. 11B) whereas NORP20 after an initial significant activation the trend slowly shifts to significant inhibition, even though they remain lesser to induce an inhibitory imbalance of NAc shell [See the color lines below in Fig. 11B indicates a significant activation (red) or inhibition (blue) of the population activity with respect to saline epoch (-30 to 0 min)]. Since it has to be noted that the induction of stereotypic behaviors was also less pronounced in NORP20 in comparison with the DEP20 and PHEN20 (Fig.7).

6.2.2 Appetite suppressant modulates all the putative cell type of NAc shell

154 well-isolated single neurons were recorded and were classified into putative Spiny Projection Neurons pSPNs (n = 41), putative Fast Spiking Interneuron's pFSIs (n = 34), putative Choline Acetyl-Transferase interneurons pChATs (n = 27) and Unidentified (n = 29) using fuzzy Sammon's mapping plot (Fig. 12A) and (see Table 3 for data regarding of firing rates and waveform shapes for each putative cell-type). With PHEN20 we recorded 59 neurons of which 16, 9, 19 and 15 are pSPN, pFSI, pChAT and Unidentified cell types respectively (Fig. 12B). Out of 60 cells recorded for NORP20 18 cells are classified as pSPN, 15 cells as pFSI, pChAT´s were 12 and 15 are Unidentified (Fig.12C).

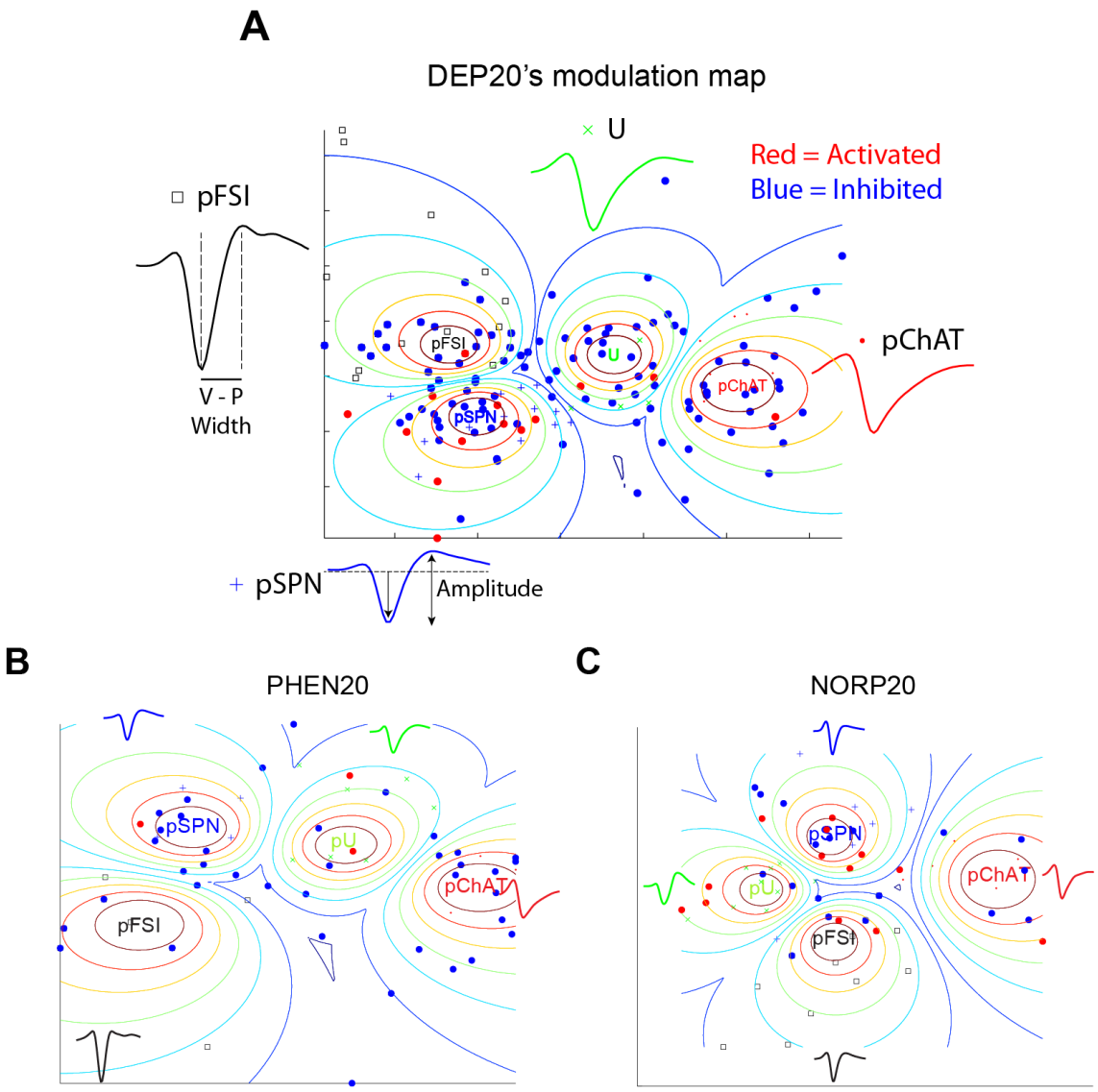

Figure 12. Appetite suppressant drugs modulates all the putative cell type of NAc shell. **(A)** A modulation map for DEP20 responses was computed with 131 neurons using a fuzzy cluster algorithm to classify them into 4 putative cell types: pSPN, medium spiny projection neuron (+); pFSI, fast-spiking interneuron (□), pChAT, choline acetyltransferase interneurons (●); and U, unidentified (×) (see Table 3). For each cell type, a representative waveform is given as well as significantly inhibited (blue dots) and activated responses (red dots). V-P is the valley-to-peak width and a waveform illustrating the amplitude. Modulation map for PHEN20 **(B)** and NORP20 **(C)** computed using the same methodology as described in panel (A).

As noted, most responses were inhibited for DEP20 and all neuronal types were equally affected ($\chi^2_{(3)} = 4.1$, $P = 0.25$, n.s., see Table 4). In contrast, for the activated responses, DEP20 significantly modulated more pSPNs than other cell-types ($\chi^2_{(3)} = 9.2$, $P = 0.02$; Table 4). We note that these activated pSPNs could be

either D1+ or D2+ expressing cells, and that they might have different sensitivities to appetite suppressants (MacAskill et al. 2014).

Table 3. Characteristics of putative cell types of NAc Shell as a function of drugs

Experiment	Waveform Shape	Putative cell types	FR (Hz)	CV2	VAR	V-P width (ms)
DEP20		pSPN (n=41)	3.18±0.37	**1.01±0.01***	0.72±0.02	0.32±0.01
		pFSI (34)	**12.07±1.68***	0.81±0.01	0.64±0.03	**0.30±0.01***
		pChAT (27)	3.96±0.41	0.95±0.02	**1.12±0.04***	0.69±0.01
		Unidentified (29)	5.69±0.90	0.85±0.02	0.90±0.02	0.53±0.01
PHEN20		pSPN (16)	2.44±0.37	**1.06±0.02***	0.73±0.02	0.28±0.01
		pFSI (9)	**7.32±1.28***	0.88±0.02	0.75±0.03	**0.25±0.03***
		pChAT (19)	3.74±0.57	0.96±0.02	**1.35±0.20***	0.66±0.01
		Unidentified (15)	3.29±0.58	1.08±0.02	0.82±0.02	0.48±0.01
NORP20		pSPN (18)	1.66±0.33	**1.08±0.01***	0.78±0.01	0.27±0.02
		pFSI (15)	**5.04±0.83***	0.85±0.02	0.75±0.03	**0.25±0.02***
		pChAT (12)	2.57±0.37	0.95±0.02	**0.85±0.19***	0.65±0.02
		Unidentified (15)	2.10±0.23	0.96±0.01	0.61±0.02	0.32±0.01

CV2, coefficient of variation 2; VAR, valley to amplitude ratio; V-P, valley to peak width. Values are mean±SEM. *$P < 0.05$, comparison across putative cell-types.

With PHEN20, all cell-types were equally inhibited (Table 4; $\chi^2_{(3)} = 2.6$, $P = 0.44$), whereas only three neurons were activated (1 = pSPN and 2 = Unidentified). NORP20, also non-significant for both inhibited and activated cell types neurons (Inhibited: $\chi^2_{(3)} = 0.9$, $P = 0.83$ and Activated: $\chi^2_{(3)} = 1.4$, $P = 0.70$).

Table 4. Neurons with a significant firing rate modulation after drug infusion as a function of cell types.

		Number of neurons			
Drug	Type of response	pSPN	pFSI	pChAT	Unidentified
DEP 20 (n=131)	Activated 14 (11%)	**10/41 (24%)#**	1/34 (3%)	1/27 (4%)	2/29 (7%)
	Inhibited 82 (63%)*	18/41 (44%)	21/34 (62%)	20/27 (74%)	23/29 (79%)
PHEN 20 (n=59)	Activated 3 (5%)	1/16 (6%)	0/9 (0%)	0/19(0%)	2/15 (13%)
	Inhibited 38 (64%)*	11/16 (69%)	5/9 (55%)	16/19 (84%)	6/15 (40%)
NORP20 (n=60)	Activated 13 (22%)	6/18 (33%)	2/15 (13%)	2/12 (17%)	3/15 (20%)
	Inhibited 18 (30%)	6/18 (33%)	4/15 (26%)	5/12 (42%)	3/15 (20%)

Values are number of neurons per indicated population within each cell-type, with percentages given in parentheses. *$P < 0.05$ comparison between activated vs. inhibited and #$P < 0.05$, comparison across all four putative cell-types.

6.3 Appetite suppressant drugs induces local field potential (LFP) oscillations in the NAc shell

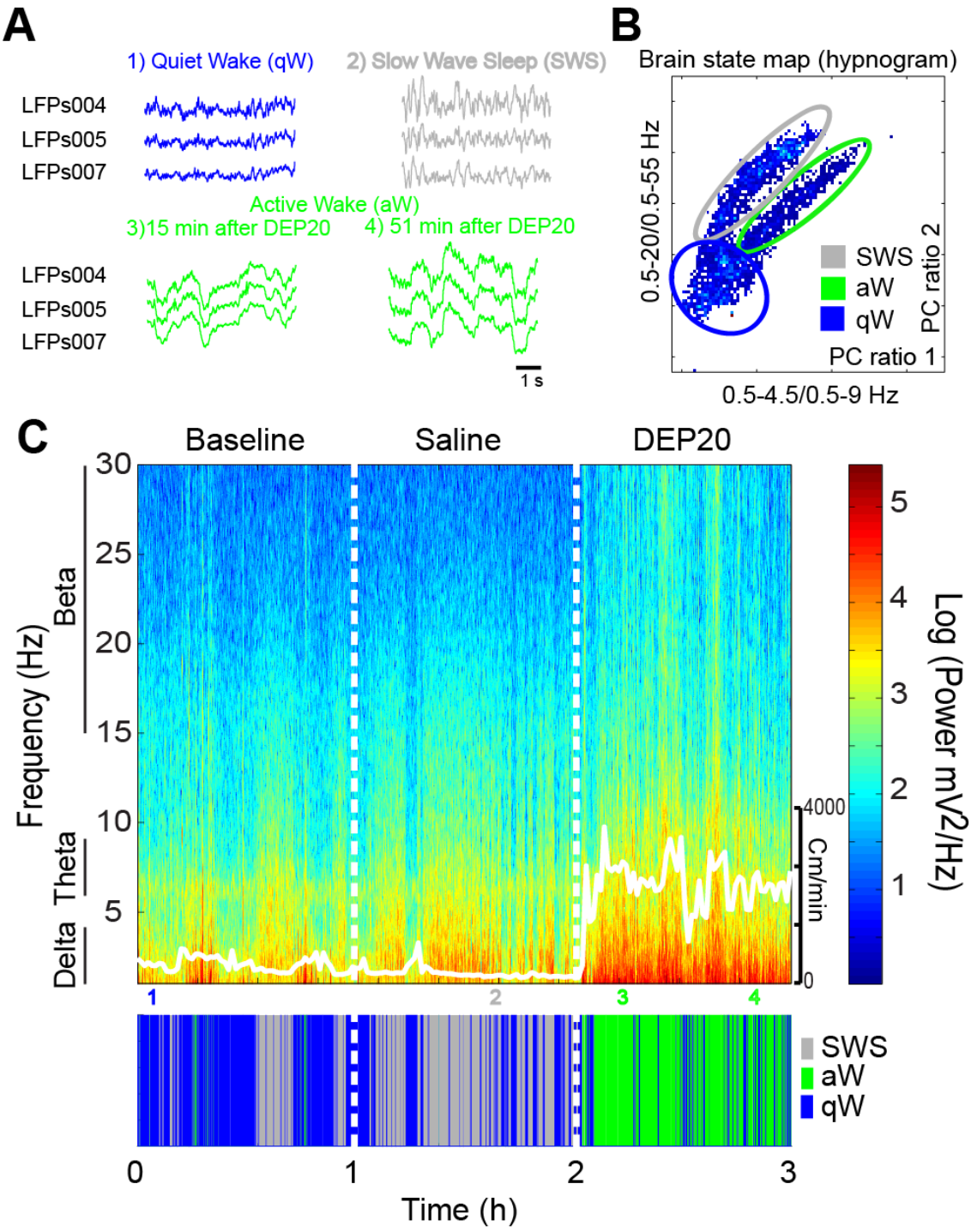

Figure 13. DEP20 increases oscillations in the NAc shell. (A) Raw traces of LFP´s recorded while an animal was in the qW (blue), SWS (gray), and aW states (green), with the latter shown 15 and 51 min after intragastric infusion of DEP20. The colored numbers indicate the times at which the raw LFP traces were obtained from the spectrogram (plotted

in (C). **(B)** A brain state map (hypnogram) was computed from the local field potentials (LFPs) in the NAc shell illustrating the 3 major behavioral states of an animal during the trial: active awake (aW), quiet awake (qW), and slow-wave sleep (SWS) (see Table 5). Each dot represents the principal component (PC) ratio between 2 power ranges for each second of LFP activity. Each dot falling into the gray ellipsoid corresponds to periods when the animals were in SWS, and the blue and green ellipsoids represent periods when the animals were in the qW and aW states, respectively. **(C)** Spectrogram of the LFPs taken from 1 to 30 Hz for the BL, Sal, and DEP20 epochs (recorded on day 1). The animal's locomotor activity is shown as a thin white trace (scale on right). Below represents the hypnogram across the session. Note that after DEP20, rats are primarily in the aW state (green). The delta power (1–4 Hz) increases during SWS (bottom) and exhibits larger amplitude after DEP20.

In addition to the recording single-unit activity we simultaneously recorded LFPs. Figure 13A shows 5 s representative LFP recordings during DEP20 session when the animals were in three distinct behavioral states. The quiet awake state (qW) is characterized by low amplitude and fast oscillations (blue traces), whereas during slow-wave sleep (SWS) they show their characteristic high amplitude low frequency delta (1-4 Hz) oscillations (gray traces). The DEP20 -induced active awake state (aW) is also characterized by high amplitude low frequency delta oscillations that over time become larger (green traces).

The (Fig.13B) brain state map (hypnogram) displays the animal's behavior of a session plotted against two frequency ranges. Each dot represents the rat´s behavior during each second, it clearly shows that animal exhibits three behavioral different states namely quiet awake (blue), active awake (green) or in slow wave sleep SWS (gray) states. Figure 13C shows a spectrogram (1-30 Hz) of the LFP's over a single session that encompasses the baseline, saline and DEP20 epochs. It is readily seen that after DEP20 infusion the power at delta, beta and theta frequency bands greatly increased. Also shown is the animal's locomotion (overlapped white line) that increased upon DEP20 application. The (Fig. 13C) panel below the hypnogram displays the animal's behavior during the three epochs, that in the baseline and saline epochs the animals were either in a quiet awake (blue), active awake (green) or in slow wave sleep SWS (gray) states (Table 5). Again, it clearly shows that in the DEP20 epoch the animal does not exhibit SWS and is primarily in the active awake state (Table 5).

Table 5. Time spent in each behavioral state across 60 min epochs.

Drug	Behavioral states	Time (min)		
		Baseline	Saline	Drug
DEP20 (n=23)	qW	20.6 ± 2.4	20.3 ± 2.1	**8.5 ± 1.3** *
	SWS	33.1 ± 2.9	32.7 ± 2.9	**0.7 ± 0.2** *
	aW	4.9 ± 0.7	5.4 ± 0.9	**48.3 ± 1.5***
PHEN20 (n=15)	qW	16.0 ± 3.6	22.1 ± 3.9	**9.7 ± 2.2** #
	SWS	35.8 ± 4.1	30.1 ± 4.3	**7.2 ± 2.1** *
	aW	7.1 ± 1.4	6.4 ± 1.4	**41.1 ± 2.8***
NORP20 (n=19)	qW	7.9 ± 0.6	9.9 ± 0.8	**3.1 ± 1.5** #
	SWS	37.9 ± 0.9	34.9 ± 1.5	**0.7 ± 0.2** *
	aW	13.4 ± 1.0	14.3 ± 1.8	**54.9 ± 1.7***

Values are mean ± SEM. * $P < 0.05$ significantly different from both baseline and saline epochs. # $P < 0.05$ significantly different from saline epoch. qW = quiet wake; SWS = Slow Wave Sleep; aW = active awake.

These three states were obtained from analysis of LFPs (Fig. 13B). In these experiments during the DEP20 epoch the animals were essentially in the active awake (aW) state (2-3 h) the entire epoch (green 48.3 min; Table 5). In this regard, after DEP20 infusion, the amount of SWS significantly decreased from 36.2 and 33.9 min in the baseline and saline epochs, respectively to 0.7 min in the DEP20 epoch (Table 5; n=23 sessions; one-way ANOVA; main effect epochs, $F_{(2, 66)} = 103.7$, $P < 0.0001$). Similarly, PHEN20 and NORP20 also significantly reduced the SWS in the animals and thereby increasing the behavior of the animals with an active awake state (PHEN20: $F_{(2, 42)} = 72.7$, $P < 0.0001$ and NORP20: $F_{(2, 54)} = 113.7$, $P < 0.0001$).

Figure 14A displays the average of 23 recording (DEP20) sessions of the normalized Power Spectral Density (PSD) of the LFPs for beta (15-30 Hz) theta (6-9 Hz) and delta (1-4 Hz) oscillations. Interestingly, there were no significant changes during the baseline or saline epochs; a result that could arise from an averaging of the quiet awake and slow wave sleep states that occur at different times across experiments. Nevertheless, after DEP20 infusion, large and rapid (~5 min) changes were revealed as a decreased z-score PSD at beta (Kruskal-Wallis; $H_{(2)} = 18412$, P

< 0.0001) and increase theta ($H_{(2)}$ = 1215, P = 0.01) and an increase in delta oscillations ($H_{(2)}$ = 5090, P < 0.0001; Table 6).

6.4 Appetite suppressant drugs-induced locomotion was also characterized by high amplitude, low-frequency delta oscillations

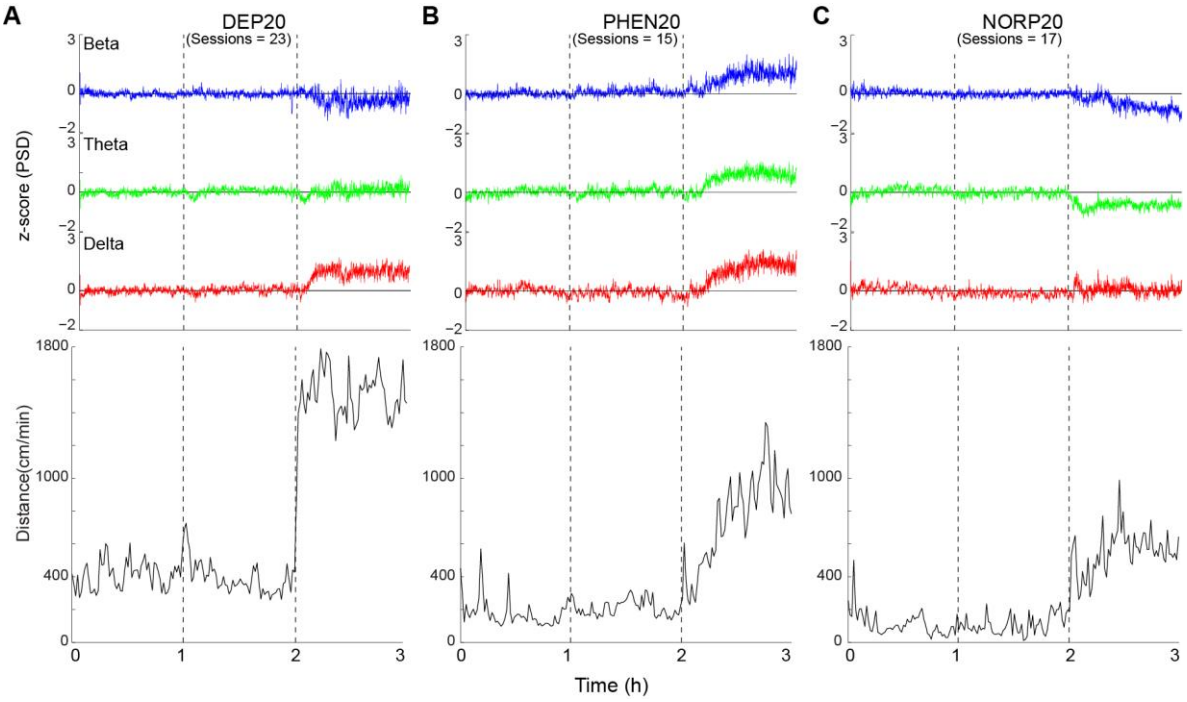

Figure 14. Appetite suppressant drugs modulates the LFP oscillations in the NAc shell. (A) Normalized and smoothed power spectral densities (PSDs) at the beta (15–30 Hz), theta (6–9 Hz), and delta (1–4 Hz) frequency bands obtained for BL, Sal, and DEP20 epochs across the entire 7-day treatment period. During the DEP20 epoch, theta and delta oscillations increased whereas beta oscillations decreased. Bottom: average locomotor activity measurements (cm/min) obtained during BL, Sal, and DEP20 epochs of all the sessions. **(B) & (C)** Top: normalized PSDs of mean LFPs at beta, theta, and delta frequencies during BL, Sal, and PHEN20 and NORP20 epochs respectively. Consistent with DEP20 both PHEN20 and NORP20 increased the LFP delta oscillations. Bottom: PHEN20 and NORP20 both increased locomotion, albeit with a delay and lesser magnitude than DEP20.

The mean locomotion responses (Fig. 14A, Bottom panel) show that compared with the saline epoch (390 ± 12 cm/min), after DEP20 infusion there is a rapid (onset: 5.43 ± 0.44 min; n=22) increase in locomotion (1502 ± 20 cm/min) (Kruskal-Wallis; $H_{(1)}$ = 89.26, P < 0.0001). In summary, these data show that DEP20 produces insomnia, increases locomotion, and alters oscillations in the NAc shell.

Table 6. Z-score power spectral density (PSD) of NAc Shell' LFP as a function of drug.

Drug	Frequency band	Z-score PSD of NAc Shell' LFP for epoch		
		Baseline	Saline	Drug
DEP20 (n=23)	Beta	-0.012±0.003	-0.002±0.007	**-0.333±0.034***
	Theta	0.015±0.003	0.088±0.004#	**0.126±0.007***
	Delta	0.013±0.003	0.069±0.004#	**0.979±0.025***
PHEN20 (n=15)	Beta	0.002±0.006	0.106±0.007#	**0.913±0.029***
	Theta	0.030±0.004	0.051±0.005	**0.863±0.007***
	Delta	0.018±0.004	-0.085±0.006#	**1.121±0.030***
NORP20 (n=17)	Beta	0.007±0.004	-0.067±0.004	**-0.614±0.029***
	Theta	0.045±0.003	-0.080±0.003	**-0.697±0.006***
	Delta	0.022±0.004	-0.201±0.004#	**0.002±0.031***

Values are mean±SEM. * $P < 0.05$, significantly different from both baseline and saline epochs. # $P < 0.05$ significantly different from baseline epoch.

With regard to the changes in LFPs evoked by PHEN20 and/or NORP20, we found both similarities and differences to those evoked by DEP20. Whereas over the entire 7 days treatment period DEP20 decreased beta and increased theta and delta oscillations, all three oscillations were increased by PHEN20 (Fig.14B). Whereas, NORP20 strongly reduced the beta and theta oscillations ($P < 0.0001$) but significantly increased their delta oscillations ($P = 0.001$) when compared with their respective saline infusion (Fig. 14C and Tables 6). With regard to locomotion (Fig. 14 B & C, bottom panels), we found that DEP20 infusions caused a greater increase in locomotion than either PHEN20 or NORP20 ($F_{(2, 54)} = 19.3$, $P < 0.0001$). Furthermore, the onset of locomotion induced by DEP20 (5.43 ± 0.44 min) was significantly shorter than for PHEN20 (19.1 ± 3.6; $P = 0.0003$) or NORP20 (10.7 ± 2.3 min; $P = 0.001$).

Our data demonstrates that appetite suppressant drugs are able to modulate the NAc shell LFP´s. Although we found difference in their modulation, all the three drugs found to induce a characteristic high amplitude low frequency delta oscillation (which has been known as a characteristic for SWS) in the NAc shell (Fig.15) but with an active awake state (insomnia).

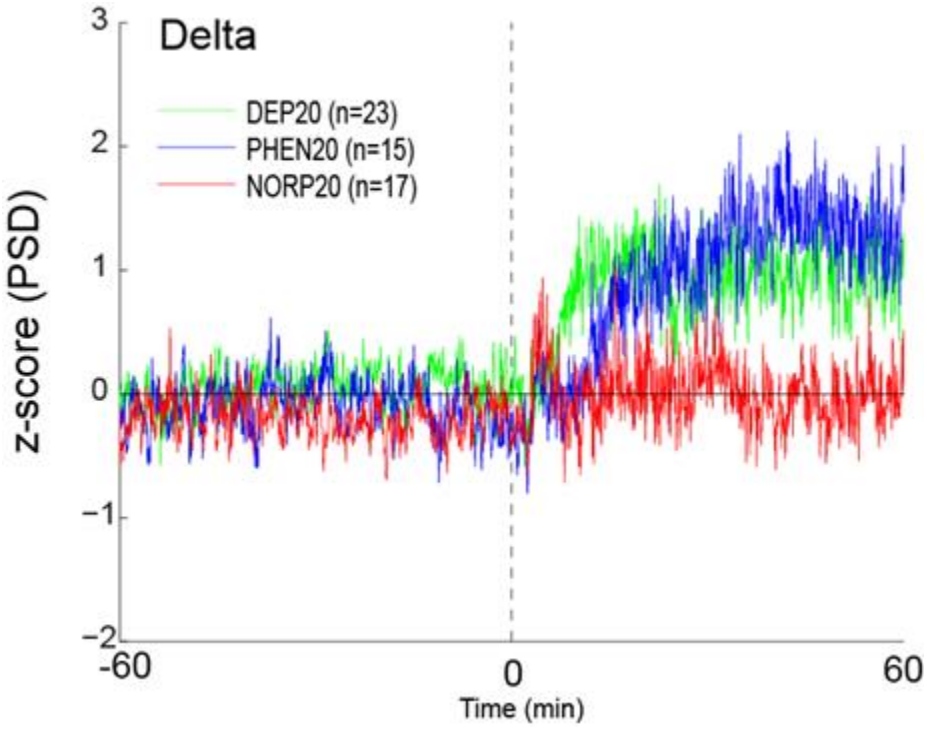

Figure 15. Appetite suppressant drugs increased the NAc shell LFP´s delta oscillations. Normalized (relative to saline time interval: −60 to 0 min) and smoothed power spectral density (PSD) at delta (1–4 Hz) frequency bands obtained during DEP20 (green traces), PHEN20 (blue traces) and NORP20 (red traces) across the entire 7-day treatment period.

In summary, repeated applications of these three appetite suppressant drugss produce weight loss, decrease food intake increase locomotion, evoke a greater percentage of inhibited than activated NAc shell responses and evoke appetite suppressant -dependent changes in LFP's oscillations (increased delta) in the NAc.

6.5 Blockade of D1R and D2R directly in the NAc shell diminishes the pharmacological effects of appetite suppressant drugs

To directly address the question of whether DA receptors in the NAc shell mediate DEP's behavioral effects, we infuse -either D1R (SCH) or D2R antagonist raclopride (RAC) - directly into the NAc shell, whereas above rats received i.g. infusion (10 min after) of DEP20 or i.p. injection (after 15 min) of PHEN20 and NORP20.

DEP

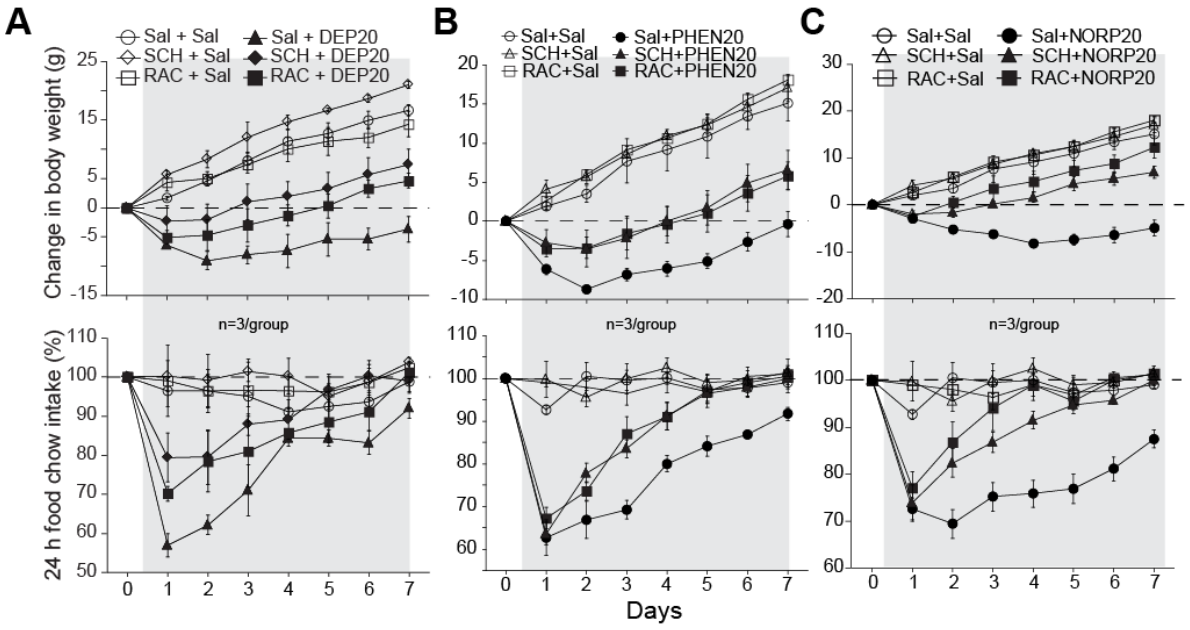

Figure 16. Intra-NAc shell infusion of either D1R (SCH) or D2R (RAC) antagonists attenuated the effect of systemic administration of appetite suppressant drugs-induced effects on weight loss and food intake. (A) The change in body weight across 7 days of treatment. Note that all groups received 2 injections: one directly in the NAc shell (Sal or SCH or RAC) and a second one intragastrically (Sal or DEP20). Thus the group Sal+Sal received saline (intra-NAc) + saline (intragastric), whereas the group Sal+DEP20 received saline (Intra-NAc) + DEP20 (intragastric), and so on for all other groups. **Bottom**: graph showing the change over 7 days in chow food intake per 24 h with the same protocol as in (A). As shown in panel (A) for DEP20, PHEN20 **(B)** and NORP20 **(C)** was also analyzed over 7-days with repeated intraperitoneal injection for the change in bodyweight (Top panels) and change in food intake (Bottom panels) after blockade of NAc shell by either D1 or D2 antagonist. Gray shading depicts either the change in body weight or 24 h food intake measured 20 min before each injection. The horizontal dotted line represents no weight change or change in food intake. Symbols represent means ± SE.

Figure 16A shows a plot of the change in body weight across seven days of daily treatment. Briefly, all groups received two injections: one directly in the NAc shell (Sal or SCH or RAC) and a second one i.g. (Sal or DEP20). A RM ANOVA showed a significant main effect of group ($F_{(5, 12)}$ = 20.4, P <0.0001), and across days ($F_{(5, 6)}$ = 87.2, P <0.0001) and significant interaction between groups and days ($F_{(30, 72)}$ = 2.7, P = 0.0004). A *post-hoc* analysis showed that relative to the control group Sal+Sal (10 ± 1.1 g); neither the group RAC+Sal (9.2 ± 1.6 g; P = 0.74) or

SCH+Sal (13.9 ± 0.9 g; $P = 0.13$) was significantly different from the control group, indicating that these DA antagonists in the NAc shell alone do not induce a significant weight loss. The weight loss produced by i.g. infusions of DEP20 was (Sal+DEP20; -6.4 ± 1.9 g; $P < 0.0001$) and it was significantly attenuated by intraNAc shell infusions of either SCH+DEP20 (positive gain weight; 2.1 ± 2.5 g; $P = 0.004$), or RAC+DEP20 (-0.8 ± 1.7 g; $P = 0.04$). Thus, D1 and D2 NAc shell receptors are clearly involved in DEP20's induction of weight loss.

For the same animals, food intake was also measured (Fig. 16A, bottom panel). Specifically, a RM ANOVA, for 7 days, showed a significant main effect of group ($F_{(5, 12)} = 7.5$, $P = 0.002$), a significant effect across days ($F_{(5, 6)} = 14.7$, $P < 0.0001$) and a significant interaction between groups and days ($F_{(30, 72)} = 3.2$, $P < 0.0001$). Overall, across seven days of treatment, a *post hoc* analysis revealed that RAC+DEP20 showed a non-significant trend to consume more chow food relative to Sal+DEP20 group ($P = 0.075$), but largely reduced chow intake in Days 2-3 (one-way ANOVA: $F_{(1, 10)} = 10.61$, $P = 0.0086$; SAL+DEP20 Vs RAC+DEP20). Likewise, the SCH+DEP20 significantly ate more food than the Sal+DEP20 group ($P = 0.009$). No differences were found between SCH+DEP20 and RAC+DEP20 ($P = 0.28$). These data show that DEP20's effects on food intake were partially, but significantly, reduced by NAc shell blockade of D1 and D2 receptors.

PHEN

Figure 16B shows a plot of the change in body weight across 7 days of daily treatment. Briefly, all groups received two infusions: one directly in the NAc shell (Sal or SCH or RAC) and a second one intraperitoneal (Sal or PHEN20). A RM ANOVA showed a significant main effect of group ($F_{(5, 12)} = 19.2$, $P < 0.0001$), and across days ($F_{(5, 6)} = 199.7$, $P <0.0001$) and significant interaction between groups and days ($F_{(30, 72)} = 3.4$, $P < 0.0001$). A *post-hoc* analysis showed that relative to the control group Sal+Sal (8.6 ± 1.1 g); the group RAC+Sal (10.2 ± 1.6 g; $P = 0.41$) or SCH+Sal (10.9 ± 0.9 g; $P = 0.45$) was not significantly different, indicating that these DA antagonists in the NAc shell alone do not induce a significant weight loss. The weight loss produced by PHEN20 was (Sal+ PHEN20; -4.8 ± 0.7 g; $P < 0.0001$) and it was

significantly attenuated by intraNAc shell infusions of either SCH+ PHEN20 (positive gain weight; 0.8 ± 1.8 g; $P = 0.02$), or RAC+ PHEN20 (0.2 ± 1.7 g; $P = 0.03$). Thus, D1 and D2 NAc shell receptors are clearly involved in PHEN20's induction of weight loss.

For the same animals, food intake was also measured (Fig. 16B, bottom panel). Specifically, a RM ANOVA, showed a significant main effect of group ($F_{(5, 12)} = 34.2$, $P < 0.0001$), a significant effect across days ($F_{(5, 6)} = 49.9$, $P < 0.0001$) and a significant interaction between groups and days ($F_{(30, 72)} = 8.4$, $P < 0.0001$). Overall, across seven days of treatment, a *post hoc* analysis revealed that RAC+PHEN20 and SCH+PHEN20 significantly consumed more chow food relative to Sal+PHEN20 group ($P = 0.0003$ and $P = 0.0006$ respectively). No differences were found between SCH+ PHEN20 and RAC+ PHEN20 ($P = 0.74$). These data show that PHEN20's effects on food intake were reduced by NAc shell blockade of D1 and D2 receptors.

NORP

Figure 16C shows a plot of the change in body weight across 7 days of treatment with NORP20. A RM ANOVA showed a significant main effect of group ($F_{(5, 12)} = 26.4$, $P <0.0001$), and across days ($F_{(5, 6)} = 122.7$, $P < 0.0001$) and significant interaction between groups and days ($F_{(30, 72)} = 8.5$, $P < 0.0001$). A *post-hoc* analysis again showed that DA antagonists in the NAc shell alone do not induce a significant weight loss effects relative to control group. The weight loss produced by NORP20 was (Sal+NORP20; -5.1 ± 0.8 g; $P < 0.0001$) and it was significantly attenuated by intraNAc shell infusions of either SCH+NORP20 (positive gain weight; 2.1 ± 1.5 g; $P = 0.0006$), or RAC+NORP20 (4.8 ± 1.7 g; $P < 0.0001$). Thus, D1 and D2 NAc shell receptors are strongly responsible for the NORP20's induction of weight loss when compared with PHEN20 and DEP20.

For the same animals, food intake was also measured (Fig. 16C, bottom panel). The percentage change in food intake was drastically attenuated by the both DA receptor antagonist. RM ANOVA, showed a significant main effect of group ($F_{(5, 12)} = 35.2$, $P < 0.0001$), a significant effect across days ($F_{(5, 6)} = 21.9$, $P < 0.0001$)

and a significant interaction between groups and days ($F_{(30, 72)} = 3.8$, $P < 0.0001$). Overall, across 7 days of treatment, revealed that RAC+DEP20 showed stronger attenuation and significantly consumed more chow food relative to Sal+NORP20 group ($P < 0.0001$), whereas SCH+NORP20 also significantly ate more food than the Sal+NORP20 group ($P < 0.0001$). No differences were found between SCH+NORP20 and RAC+NORP20 ($P = 0.07$, n.s). These data show that out of these three appetite suppressants, NORP20's anorectic and appetite suppressant effect were markedly reduced by NAc shell blockade of D1 and D2 receptors.

Here we demonstrate for the first time that DA receptor of NAc Shell is necessary for anorectic effect of these (noradrenergic) appetite suppressant drugs. This class of appetite suppressant drugs depends on both D1 and D2 receptor of NAc Shell to induce weight loss and food intake suppression (NORP>PHEN≥DEP).

6.5.1 Blockade of D1R and D2R directly in the NAc shell diminishes the secondary effects of appetite suppressants

Locomotion was also measured under the same conditions described above for the respective drugs (Fig. 16, see methods). We found that relative to Sal+Sal (381 ± 79 cm / 5 min) NAc Shell infusion of RAC+Sal (404 ± 90 cm / 5 min) or SCH+Sal (347 ± 71 cm / 5 min) do not alter locomotion. In contrast (Fig.17A), Sal+DEP20 induced a rapid onset (5 min) and robust locomotion (3303 ± 368 cm / 5 min; RM ANOVA; main effect groups $F_{(5, 118)} = 159.7$, $P < 0.0001$). For the RAC+DEP20 experiments the onset of locomotion induced by DEP20 was delayed by 15 min and the magnitude of locomotion was also attenuated (2094 ± 199 cm / 5 min; $P < 0.0001$). Similar results were found for the SCH+DEP20 experiments, the onset of locomotion induced by DEP20 was further delayed by 35 min as well as its magnitude (881 ± 47 cm / 5 min; $P < 0.0001$).

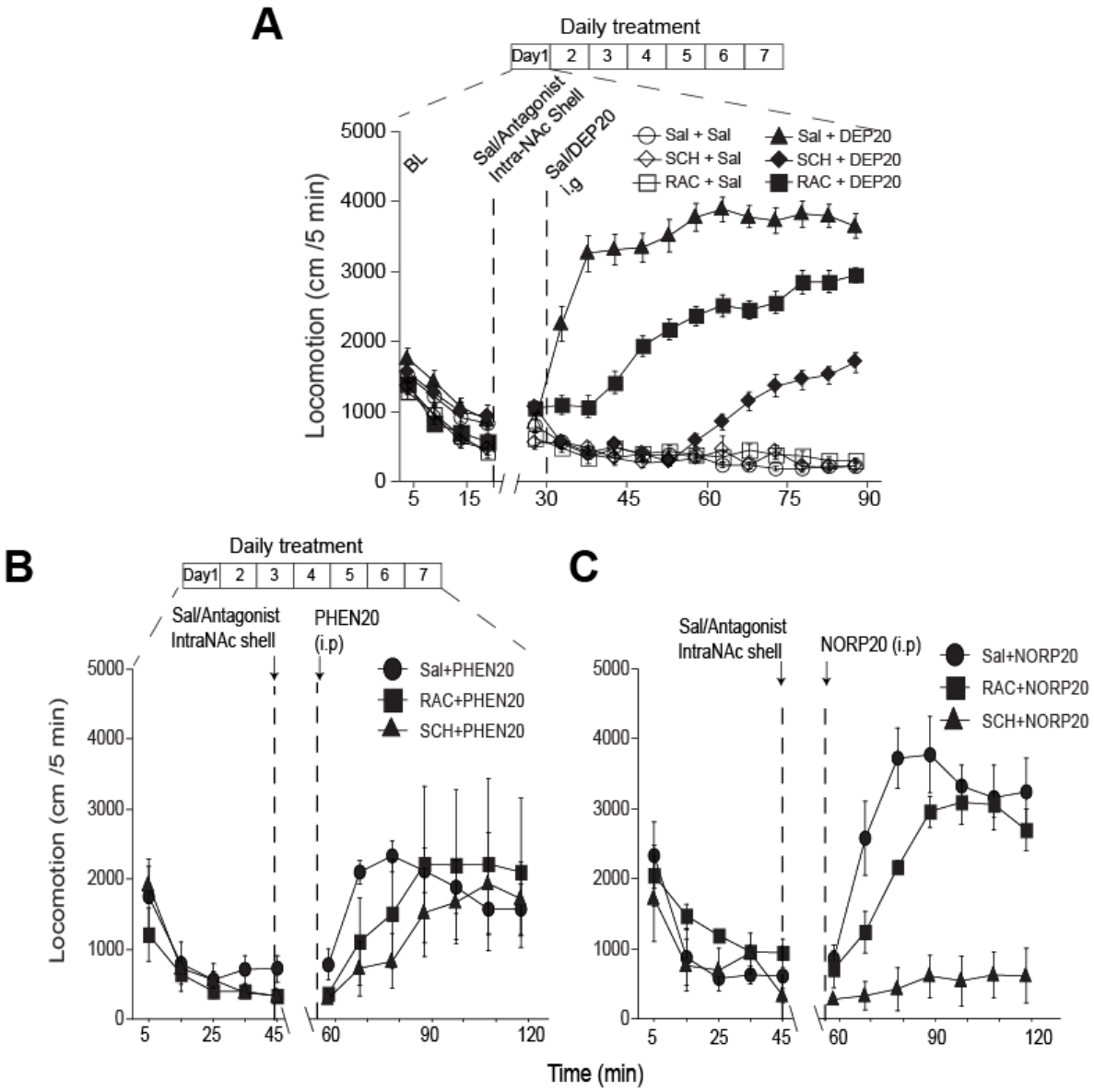

Figure 17. Intra-NAc shell infusion of either D1R (SCH) or D2R (RAC) antagonists attenuated the effect of systemic administration of appetite suppressant drugs-induced effects on locomotion. (A) Effect of SCH and RAC antagonists on locomotion measured in the open-field arena, in the same subjects shown in Figure16. At 20 min, animals were briefly removed from the open field and received an intra-NAc infusion; note the interruption in the x-axis at 20–25 min. At 30 min, all animals received the corresponding intragastric infusion. **(B)** Graph plotting locomotion for 120 min across 7-days of treatment with or without D1 or D2 antagonist and PHEN20. Note that after a brief baseline for 45 min animals were removed from the open field arena and infused with either Sal or SCH or RAC as intra-NAc shell and later at 60 min PHEN20 was injected as i.p. **(C)** Using the similar protocol as in (B) locomotor effect of intraperitoneal administration of NORP20 over 7 days was graphed, with or without blocking the NAc shell by D1R and D2R antagonist. Symbols represent means ± SE over 7-days treatment.

Similar locomotion effects to those found for DEP20 were replicated for PHEN20 and NORP20 (Fig. 17B & C respectively) but with little modification of the protocol. As mentioned above after 15 min of intraNAc infusion of DA antagonist, drugs (either PHEN20 or NORP20) were injected as i.p not as i.g. As we found that RAC or SCH by themselves did not evoke any locomotion effect (Fig. 17A). On administration of PHEN20 eventually increased their locomotion whereas blocking of either D1 or D2 antagonist of NAc shell only attenuate the PHEN20 induced locomotion for just 10 min later it induces a similar induction of locomotion which was evident by RM-ANOVA across groups ($F_{(2, 6)} = 0.42$, $P = 0.79$, n.s). Perhaps blocking of NAc shell with DA receptor antagonist blocked the pharmacological effects (Fig. 16B) induced by PHEN, here our results implies that NAc shell DA receptors are just responsible for the induction of locomotion induced by PHEN20.

Whereas NORP20 evokes a robust locomotion (RM ANOVA main effect of groups $F_{(2, 6)} = 32.8$, $P = 0.0006$), which was significantly diminished (but not eliminated) by SCH+NORP20 ($P = 0.0002$). RAC+NORP20 significantly delayed the onset of locomotion by 15 min ($P = 0.03$) later there was no difference from Sal+NORP20 which results in non-significant trend ($P = 0.06$). Importantly, SCH+DEP20 almost completely attenuated the NORP20-induced locomotion.

Hence, these results provide evidence for the involvement of NAc shell dopamine receptors (D1 > D2 receptor antagonist) in the ability of appetite suppressants to trigger and to maintain locomotion.

6.6 DEP –induced locomotion, stereotypy, weight-loss and food intake are attenuated by intragastric infusion D1R and D2R antagonists

Since the NAc shell receives dopaminergic input from various sources (Haber et al. 1985), we determined if the locomotion, stereotypy, weight-loss and food intake effects evoked by DEP20 would be modulated by i.g. infusion of either/or the D2R antagonist Raclopride 0.5 mg/Kg (RAC0.5) and/or the D1R antagonist SCH23390 1.5 mg/Kg (SCH1.5). Figure 18A shows a plot of the change in body weight in naïve rats over seven days of daily treatment (n = 3, per group). As controls, we found that relative to saline, infusions of RAC0.5 (positive gain 18.4 ± 5.4 g) or SCH1.5 (11.8 ±

2.7 g) alone do not induce a significant weight loss although; i.g. infusions of DEP20 produce a large weight loss (-15.3 ± 3.1 g). This weight loss could be reversed by i.g. infusions of either DEP20+RAC0.5 (positive gain weight; 1.4 ± 1.9 g), or DEP20+SCH1.5 (-4.4 ± 5.4 g). RM ANOVA showed a significant main effect of group ($F_{(5, 12)} = 11.4$, $P = 0.0003$), and across days ($F_{(5, 6)} = 36.4$, $P < 0.0001$) and significant interaction between groups and days ($F_{(30, 72)} = 4.9$, $P < 0.0001$). A *post-hoc* analysis showed in comparison to DEP20 both groups DEP20+RAC0.5 and DEP20+SCH1.5 (only for days 3-8) significantly attenuated the weight loss induced by DEP20 (All $P's < 0.05$).

For these same animals the percentage change in 24 h food intake was also measured (Fig. 18B). Relative to day 1, the food intake over the treatment was: Saline (4.9 ± 9.8%), RAC0.5 (5.7 ± 8.3%), SCH1.5 (2.54 ± 5.7%), DEP20 (-28.7 ± 3.8%), DEP20+SCH1.5 (-16.9 ± 7.3%) and DEP20+RAC0.5 (-7.13 ± 6.8%) (RM ANOVA showed a significant main effect of group $F_{(5, 12)} = 4.8$, $P = 0.011$ and across days ($F_{(5, 6)} = 5$, $P = 0.0002$) and significant interaction between groups and days ($F_{(30, 72)} = 2.9$, $P = 0.0001$). The DEP20+SCH1.5 group tended to eat slightly more food than DEP20 group, although the differences were not significant ($P = 0.21$), whereas RAC0.5 significantly reversed food intake suppression induced by DEP20 ($P = 0.032$; comparison DEP20 vs. DEP20+RAC0.5). These data reveal that DEP's effect on weight loss and food intake is markedly affected by inhibition of both D1 and D2 receptors. As described above (with intracranial administration experiments) that some of these effects are the result of inhibiting DA receptors in the NAc shell.

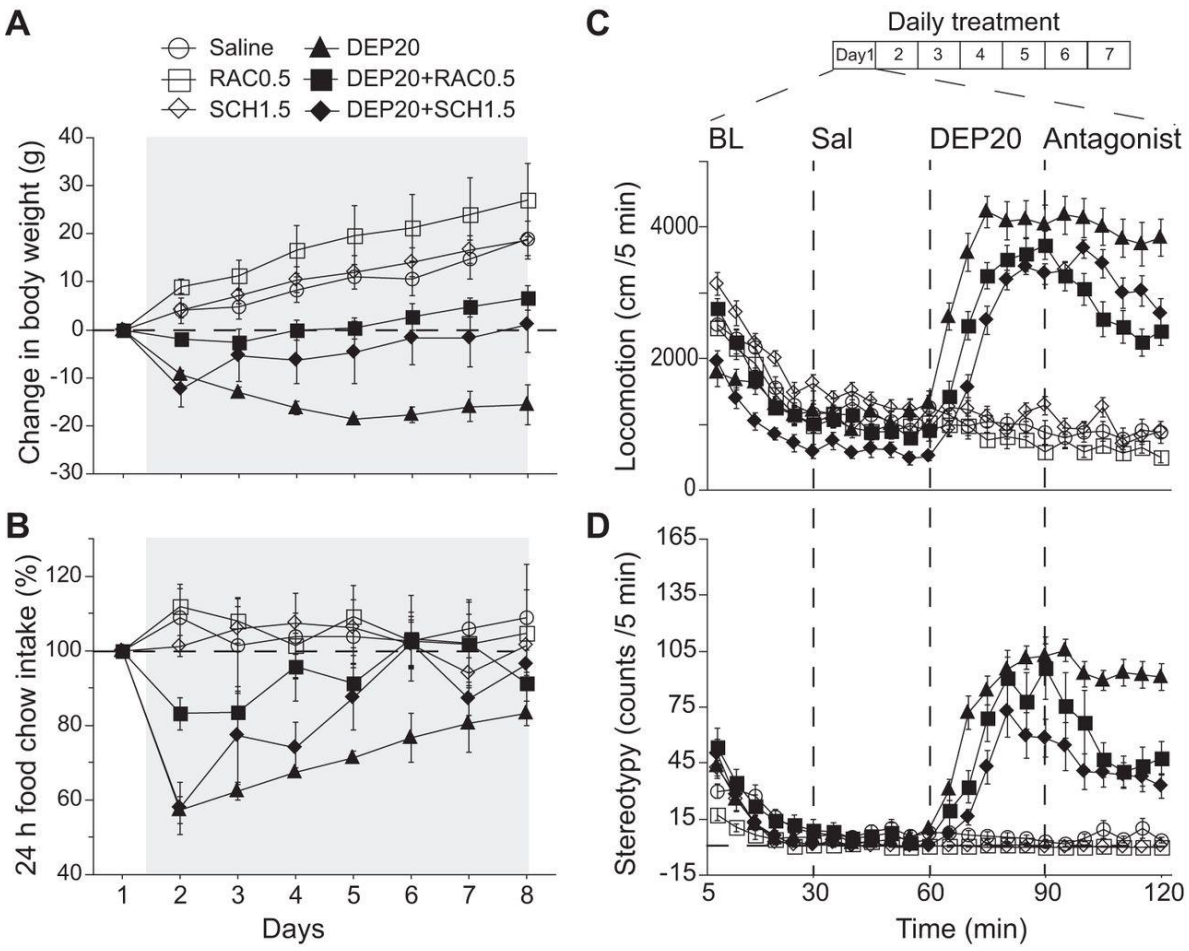

Figure 18. Intragastric D1 dopamine receptor (D1R) antagonist SCH-23390 (SCH1.5) and D2R antagonist raclopride (RAC0.5) attenuate DEP20's effect on weight loss, food intake, locomotion, and stereotypic head movements. (A) The change in body weight during 7 days of treatment with daily intragastric infusions of Sal (○), RAC0.5 (□), SCH1.5 (◇), DEP20 (▲), DEP20+RAC0.5 (■), and DEP20+SCH1.5 (⧫) starting on day 1 and terminating on day 7 (gray shading). The weight loss produced by DEP20 was reduced in the presence of either RAC0.5 or SCH 1.5. Note that neither D2 (□) nor D1 (◇) antagonists alone induced weight loss. **(B)** Graph showing the change in 24-h food intake under the same conditions as in A. Infusion of DEP20+RAC0.5 or DEP20+SCH1.5 increased food intake compared with DEP20 alone. **(C)** Effect of these DA antagonists on locomotion in the same subjects and grouping as in (A) measured in an open-field arena. All groups displayed a gradual decay in exploratory activity within 20 min from introduction to the open field (BL). Intragastric infusions were then made at 30-min intervals with saline, at 60 min with DEP20, and at 90 min with and/or without RAC0.5 or SCH1.5. Compared with DEP20 alone, repeated administrations of DEP20+RAC0.5 or DEP20+SCH1.5 decreased the magnitude and delayed the onset of locomotion. **(D)** Graph showing the reduction in the stereotypy induced by DEP20+RAC0.5 or DEP20+SCH1.5.

We also measured the animals changes in locomotion and stereotypy during a 2 h period (Fig. 18C each epoch being 30 min; baseline (BL), saline (Sal), DEP20 and antagonist). For locomotion, infusion of RAC0.5 (812 ± 137 cm / 5 min) or SCH1.5 (1,161 ± 152 cm / 5 min) alone does not increase locomotion relative to saline (1,008 ± 181 cm / 5 min). In all cases the animal's locomotion decreased from the baseline (BL) to saline epoch and increased upon the infusion of DEP20 (Note that the responses to DEP20 are not identical, likely because they are affected by the previous treatment days). The DEP20-induced locomotion (3,957 ± 293 cm / 5 min; RM ANOVA, from 1-120 min; main effect groups $F_{(5, 118)} = 45.1$, $P < 0.0001$) was attenuated by subsequent administrations of either SCH1.5 (3,193 ± 198 cm / 5 min; $P < 0.0001$; group DEP20+SCH1.5) or RAC0.5 (2,675 ± 233 cm / 5 min; $P < 0.0001$; group DEP20+RAC0.5). Locomotion was not significantly different between DEP20+RAC0.5 and DEP20+SCH1.5 ($P = 0.06$ n.s.).

Similar effects to those above were also found for stereotypy (Fig. 18D). Again application of RAC0.5 or SCH1.5 alone does not evoke head weaving stereotypy (Fig. 18D). However, DEP20 evoked stereotypy (97 ± 7 counts / 5 min; RM ANOVA, from 1-120 min; main effect groups $F_{(5, 118)} = 64.8$, $P < 0.0001$) was significantly reduced by either DEP20+RAC0.5 (54 ± 12 counts / 5 min; *Post-hoc* $P = 0.0004$) or DEP20+SCH1.5 (40 ± 8 counts / 5 min; $P < 0.0001$). Note that DEP20 –induced stereotypy remained at high levels for times longer than 1 h, whereas stereotypy declined within 30 min in the presence of the antagonists. These results provide evidence for the involvement of DA receptors in the DEP20 -induced locomotion, stereotypy as well as on its anorectic effects.

6.7 DEP20-induced LFP oscillations and spiking inhibition in the NAc shell are mediated by D1R and D2R receptors

Having shown that both i.g and intraNAc shell infusions affects behavior for technical reasons (to avoid drift in waveform stability induced by intraNAc microinjections) we only determined if i.g. infusion of the two tested DA antagonists would similarly affect DEP20 induced NAc shell activity, we recorded the

electrophysiological responses over seven days of an i.g. infusion of RAC or SCH 30 min after the infusion of DEP20 (see below for controls).

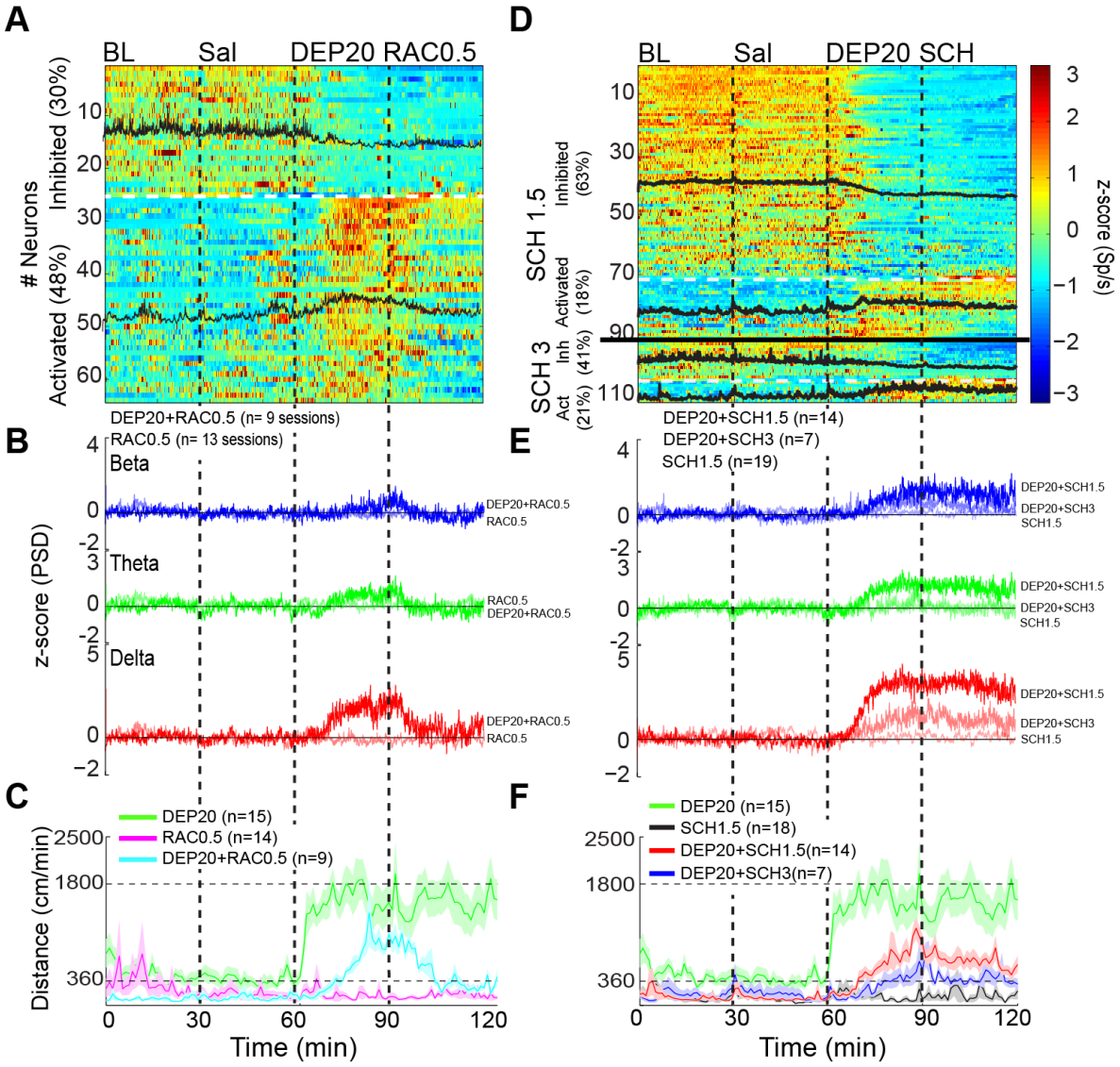

Figure 19. Intragastric dopamine D1R and D2R antagonists reverse the DEP20-induced electrophysiological changes in the rat's NAc shell. (A) A plot of the activity of 64 (of 81) NAc shell neurons that were significantly inhibited (30%) or activated (48%) by DEP20 across 7 days of chronic treatment with RAC0.5. Each of the 4 epochs was 30 min long. Note that compared with DEP20 alone (Fig. 10A), the percentage of neurons inhibited during the DEP20 epoch in the presence of chronic treatments with RAC0.5 decreased and the percentage activated increased. The black traces are the mean activity of the 2 respective categories. **(B)** The grand average of 9 experiments showing the normalized PSD at delta frequency from the same experiments shown in (A) overall days. Infusion of RAC0.5 greatly diminished the changes in all DEP20-induced oscillations. Note that infusion of RAC0.5 alone (thin traces) did not change LFP oscillations. **(C)** Locomotion increases evoked by

DEP20 were also decreased by RAC0.5 (cyan trace), whereas RAC0.5 alone did not increase locomotion (pink trace). **(D)** Color coded PSTH showing repeated administration of two doses of D1R antagonist (SCH1.5 and SCH 3) infused after DEP20. The firing rate modulation of 91 out of 112 (and 21 out of 34) single neurons was recorded in the NAc shell during 30-min BL, Sal, DEP20+SCH1.5, and DEP20+SCH3 infusions, respectively. Under repeated administration SCH, DEP20 did not produce a robust spiking inhibition [SCH1.5 (63%) and SCH3 (41%) compared with DEP20 alone (63%, 82/131)]; rather, it increased its activation [SCH1.5 (18%) and SCH3 (21%) compared with DEP20 alone (11%, 14/131)]. **(E)** A plot of the LFP PSD (top) induced by SCH1.5 (red trace), SCH3 (blue trace), and SCH1.5 alone (black trace). D1R antagonist attenuated in a dose-dependent manner the DEP20-induced delta oscillations. Note all oscillations are positive. The infusion of SCH1.5 alone (thinnest lines) did not change LFP oscillations, with values around 0 z-score. **(F)** In a dose dependent manner D1 antagonist delayed the onset and diminished the magnitude of locomotion evoked by DEP20.

Figure 19A is a plot of the normalized color-coded PSTHs of all neurons (over 7 days) modulated by DEP20 during the Baseline (BL), Saline (Sal), DEP20 and RAC0.5 epochs. In the presence of RAC0.5 (DEP20+RAC0.5) the extreme bias towards the inhibition seen with DEP20 alone (over 7 days) were diminished since now only 30% (39/81) were inhibited and 48% (25/81) were activated ($\chi^2_{(1)} = 2.2$, $P = 0.13$, n.s.). The averaged normalized DEP20-induced z-score PSD for the beta, theta and delta oscillations over the treatment period are seen to decrease to baseline levels by RAC0.5 (Fig. 19B*).* Finally, RAC0.5 alone did not induce changes in the LFP's oscillations at beta, theta and delta bands over the 7 days of treatment (see Fig. 19B thinner traces), and neither induced locomotion during single-unit recordings (Fig. 19C bottom panel, pink trace).

RAC0.5 also attenuated and delayed the DEP20 –induced increase in locomotion (Fig. 19C, bottom panel; cyan trace locomotion measured in the operant chamber; Kruskal-Wallis, $H_{(2)} = 53$, $P < 0.0001$) and delayed the onset of DEP20-induced locomotion by 11.8 ± 2.2 min, which was significantly slower to that normally evoked by DEP20 alone (6.2 ± 1.36 min; $F_{(1, 21)} = 5.05$ $P = 0.035$).

We also tested SCH1.5 alone and found that it modulated single-unit activity to about the same extent inducing nearly the same amount of inhibition (n = 34/114 neurons, 29.8%) as activation (n = 35/114 neurons, 30%; Data not shown). Accordingly, SCH1.5 alone does not produce any inhibitory imbalance at the population activity level (see Fig. 19D, black trace). In this regard, SCH1.5 did not

restore the DEP20-induced inhibitory imbalance such that with SCH1.5 there were 63% (71/112) inhibited and 17.8% (20/112) activated (χ^2= 20.8, P < 0.0001; Fig. 19D) neurons. This result was unexpected given the effect of SCH1.5 on the behavioral data (Fig. 18, DEP20+SCH1.5). Consequently, we determined if SCH3 would restore the DEP20-induced imbalance. Indeed, under this condition 41% (14/34) were inactivated and 21% (7/34) activated (the proportions of inactive/active responses were not significantly different; χ^2= 1.7, P = 0.18 n.s.). Thus, SCH3 effectively rebalanced the DEP20-induced imbalance (inhibited/activated) by decreasing the number of neurons with inhibited responses and slightly increasing the number of active responses.

With regard to the DEP20-induced beta, theta and delta oscillations over the treatment period, it is seen that the onset of DEP20-induced oscillations were markedly delayed by SCH1.5 and even more for SCH3 (Fig. 19E). As a control we found that SCH1.5 alone did not modulate beta, theta or delta LFP's oscillations (Fig. 19E, thinnest traces). In addition, after the infusion of SCH1.5, the magnitude of the LFP's PSD slightly changed (from 3.0 ± 0.08 in the DEP20 epoch to 2.59 ± 0.08 in the SCH epoch). Likewise, for SCH3, it also marginally changed from 1.18 ± 0.06 in the DEP20 epoch to 0.92 ± 0.07 in the SCH1.5 epoch. Nevertheless, SCH markedly attenuated DEP20-induced beta, theta and delta oscillations in a dose-dependent manner. Likewise, SCH decreased the locomotion in a dose dependent manner. It also delayed the onset of DEP20-induced locomotion from 6.2 ± 1.4 min to 11.5 ± 2.5 min (SCH1.5) and 24.9 ± 4.5 min (SCH3) ($F_{(2, 33)}$ = 11.64, P = 0.0001, green; Fig. 19F).

Figure 20A shows the global population-firing rate of all 81 neurons recorded with DEP20+RAC0.5 (cyan). It is seen that the global inhibitory imbalance induced by DEP20 is largely abolished by repeated administrations of RAC0.5. Nevertheless, RAC0.5 alone modulated NAc shell single-unit activity although the percentage of neurons inhibited (n=29/93, 31%) was similar to the percentage of those activated (n= 26/93, 28%) (*P = 0.72, n.s.*).

6.8 Systemic infusion of D1 and D2 antagonist reversed the global inhibition of NAc shell spiking activity induced by DEP

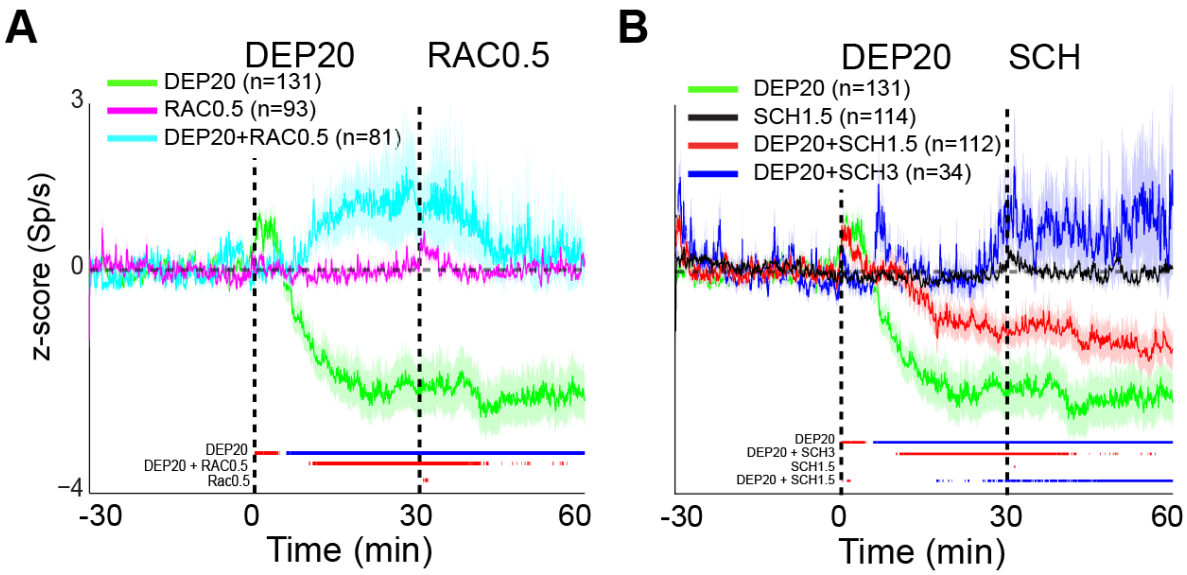

Figure 20. Intragastric dopamine D1R and D2R antagonists reverse the DEP20-induced inhibition/activation imbalance in the rat's NAc shell. (A) A plot of the normalized global population responses of all neurons recorded (n = 131) with DEP20 alone (green; same data as in Fig. 10A, aligned to DEP injection time = 0 min) vs. all 81 neurons recorded with DEP20 and chronic treatment with RAC0.5 (cyan trace over same treatment days, DEP20+RAC0.5). Note that after the infusion of RAC0.5, the population firing rate returned to near baseline levels. Finally, the infusion of RAC0.5 alone did not produce any inhibitory imbalance (pink trace). The colored lines at bottom indicate the bins (1-min resolution) with significant increases (red) or decreases (blue) of population activity relative to saline firing rates (time interval: −30 to 0 min). **(B)** The average firing rate of all neurons recorded under repeated treatment of DEP20 followed by SCH1.5 (red trace) and SCH3 (blue trace) and treatment of SCH1.5 alone (black trace). Note that SCH in a dose-dependent manner reversed the inhibition/activation imbalance induced by DEP20.

In summary, during the infusion of RAC0.5 alone the average population activity of all 93 neurons recorded showed a slight but transient activation that rapidly returned to baseline (< 4 min; see Fig. 20A, pink trace). It follows that RAC0.5 alone does not induce an inhibitory imbalance in the NAc shell population activity. Finally, to determine at the population firing rate level whether SCH would reversed the inhibition induced by DEP20, we plotted in Figure 20B the population activity of all the recorded neurons (modulated or not by DEP20). It is seen that SCH reversed the strong inhibition evoked by DEP20 in a dose-dependent manner at the population NAc shell activity level.

6.9 Behavioral and neuronal correlates for the mechanism of appetite suppressant drug.

6.9.1 DEP20-induced changes in NAc shell activity across treatment period

Having shown that repeated i.p. injections of DEP20 produce transient changes in weight loss and food intake (Fig. 7A), we investigated if activity changes measured in the NAc shell would correlate with these behavioral changes. To achieve this, over 14 days, we gave daily i.g. infusions of saline and DEP20 while measuring the NAc shell activity. Animals given the i.g. treatment, like those given the i.p. treatment, showed a transient increase in weight loss and decrease in food intake that adapted as the treatment progressed (Fig. 21A). To obtain a sufficient number of neurons for statistical analysis the treatment was divided into three periods (Fig. 21A): day 1, days 2-7 and days 8-14. The mean of the population firing rates and normalized firing rates for day 1 (purple), days 2-7 (orange) and days 8-14 (brown), over the baseline, saline and DEP20 epochs are shown in Figure 21B and Table 7. Throughout the three epochs the greatest activity (baseline and evoked) occurred in days 2-7 and the least were in days 8-14. The normalized responses revealed no differences between the baseline and saline epochs and that the greatest inhibitory effect was during days 8-14 (Table 7).

Analysis of the LFP oscillations over these three periods shows dramatic changes in DEP20 responses (Fig. 21C, Table 7). Specifically, on day 1 there were statistically significant increases at beta, theta and delta oscillations. On days 2-7 all three oscillations decreased in magnitude and on days 8-14 the beta and theta oscillations became largely negative and the delta oscillations decreased to saline levels. Thus, the changes in weight loss and food intake are best correlated with increases in the oscillations on day 1 and the adaptation (tolerance) to the treatment is better correlated with the diminution of the amplitude of the oscillations over the succeeding treatment days.

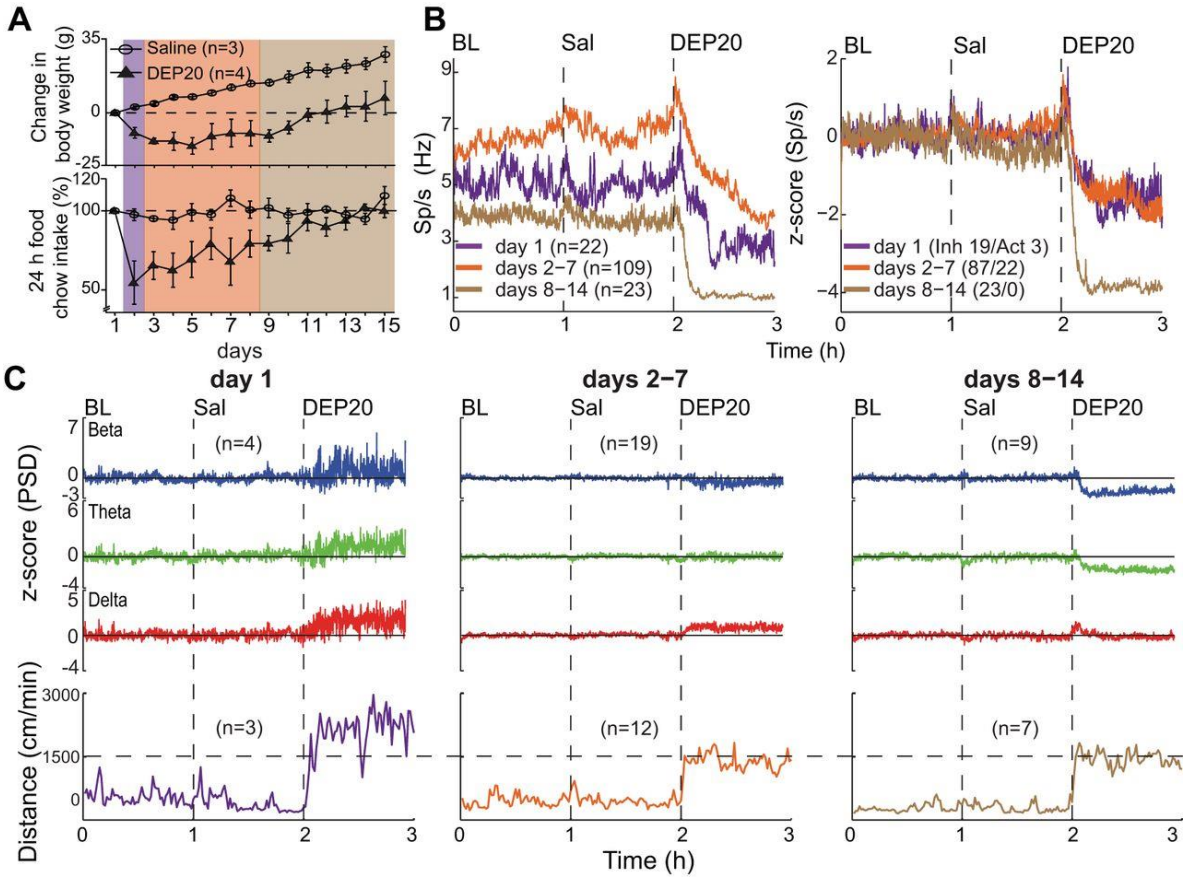

Figure 21. Behavioral and neuronal changes of NAc shell activity across 14 days of DEP20 treatment. (A) Plots of weight loss and food intake over 14-day treatments of saline or DEP20. With intragastric saline the animal's weight monotonically increased across days, whereas for DEP20, after a decrease that lasted until day 9, the animals gained weight at the same rate as the saline-treated animals. The DEP20 group transiently reduced food intake and then increased it with increasing treatment days. Purple shading indicates day 1, orange days 2–7, and brown days 8–14. **(B)** Left: a plot of the neuronal population changes observed across repeated intragastric DEP20 infusions. The colors codes are the same as in A. Relative to treatment day 1, during days 2–7, DEP20 increased global firing rates during BL and Sal epochs, whereas after DEP20 for days 8–14, the activity decreased. For days 8–14 relative to baseline, the activity is lower in all 3 epochs. Right, normalized activity in the BL, Sal, and DEP20 epochs. The latter reveals a much larger inhibition during days 8–14. The ratios of inhibitory to activated responses are also presented. **(C)** Top: changes in the LFP PSDs at beta, theta, and delta frequencies across days. All DEP20-evoked oscillations were positive at day 1, but all decreased across days, with the beta and delta oscillations becoming negative at days 8–14. Bottom, locomotion evoked by DEP20 across days.

Table 7. Behavioral and neuronal changes on NAc shell activity after repeated use of DEP20.

Drugs	Periods	Relative to day before treatment		Activity		Baseline	Saline	Drugs
		Change in bodyweight (g)	24 h chow intake (%)					
DEP20	Day 1	- 9.5 ± 2.5	- 45.4 ±13.8	Firing rate (Hz)	All (n=22)	5.1±0.02	4.9±0.01	3.0±0.02
					Inhibited (19)	5.4±0.01	5.4±0.01	1.9±0.04
					Activated (3)	2.4±0.01	2.8±0.01	4.6±0.03
				z-score (PSD)	Beta (15-30Hz)	0.030±0.009	0.033±0.008	0.638±0.009
					Theta (6-9 Hz)	0.042±0.008	0.187±0.008	1.063±0.012
					Delta (1-4Hz)	0.020±0.009	0.088±0.008	1.519±0.007
				Distance (cm/min)		555±24	383±26	2088±64
	Days 2-7	- 12.3 ± 1.9	- 29.4 ± 4.2	Firing rate (Hz)	All (109)	6.3±0.01	6.6±0.01	4.5±0.03
					Inhibited (87)	5.6±0.01	6.0±0.01	2.0±0.04
					Activated (22)	3.1±0.01	3.0±0.04	7.9±0.18
				z-score (PSD)	Beta	-0.017±0.003	-0.007±0.008	**-0.455±0.035** *
					Theta	0.012±0.003	0.066±0.004	**0.008±0.008** *
					Delta	0.013±0.003	0.063±0.004	**0.918±0.026** *
				Distance (cm/min)		452±19	448±16	1382±25
	Days 8-14	**- 1.8 ± 2.1***	**- 9.6 ± 2.9***	Firing rate (Hz)	All (23)	3.8±0.003	3.7±0.006	1.1±0.003
					Inhibited (23)	3.8±0.003	3.7±0.006	1.1±0.003
					Activated (0)	-	-	-
				z-score (PSD)	Beta	-0.007±0.005	0.001±0.006	**-1.574±0.019** *
					Theta	0.028±0.004	-0.008±0.005	**-1.459±0.008** *
					Delta	0.029±0.004	-0.069±0.005	**-0.047±0.017** *
				Distance (cm/min)		280±12	294±14	1449±24

Values are mean ± SEM. * $P < 0.05$, comparison across periods

6.9.2 DEP20 induces taste aversion to a novel taste (similar to LiCl), but the NAc shell LFP's oscillations induced by DEP20 are different than those evoked by LiCl.

In order to explore whether DEP20 has a potential to induce taste aversion, which may contribute to its anorectic effects, we performed a conditioned taste aversion (CTA) experiment. We used DEP20 or LiCl (0.4 M), as unconditioned stimuli (US) and a novel taste (saccharin) was the conditioned stimulus (CS). Figure 22A displays the 15 min intake of water and the CS across the experiment, note that during 3 days of baseline (W1-3), water intake gradually increased as rats habituate to drink their entire daily allotment of fluids within 15 min. On the acquisition day (ACQ) the animals were presented with a novel 0.1% saccharin solution in which they consumed less saccharin than water on W3 day. Surprisingly, in the TEST session, both DEP20 and LiCl groups rejected saccharin to the same extent (one-way ANOVA: $F_{(1, 10)} = 1.3$, $P = 0.28$), suggesting that they were able to associate a

gastric distress induced by a single injection of DEP20 with the consumption of a novel taste.

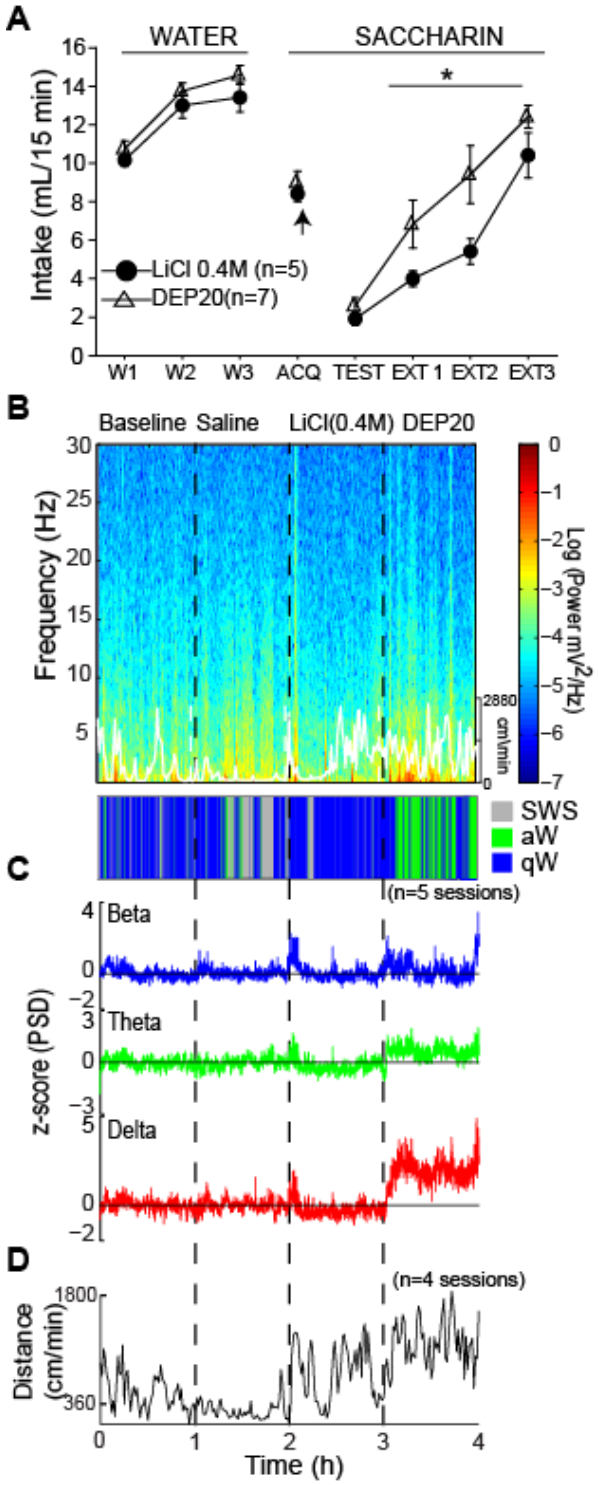

Figure 22. DEP20 induces conditioned taste aversion to a novel 0.1% saccharin solution, but DEP20-induced LFP oscillations are unrelated to an aversive malaise brain state.

(A) The daily 15-min intake (in ml) of water (W1–W3) and sodium saccharin (ACQ, Test, and EXT1–EXT3). The arrow (acquisition day, ACQ) indicates the time of injection of LiCl or DEP20, 15 min after consumption of the novel saccharin. The Test session occurred 72 h after ACQ; note that both groups rejected saccharin at similar levels, but during the 3 extinction days (EXT1–EXT3), DEP20 extinguishes the taste aversion faster than LiCl (*P <0.05, RM ANOVA). (B) Spectrogram (top) of the NAc shell LFP for the BL, Sal, LiCl (0.4 M), and DEP20 epochs, with corresponding hypnogram (bottom). The white trace shows the animal's locomotor activity (scale on right). (C) Normalized PSD at the beta (15–30 Hz), theta (6–9 Hz), and delta (1–4 Hz) frequency bands across the experiment. D: average locomotor activity (in cm/min) obtained during each epoch shows that LiCl upsets the animal, resulting in distress movements but not stereotypy (not shown) and exploratory locomotion.

This is reflected by the smaller intake of saccharin in the TEST session compared to that in the ACQ day. Nevertheless, as measured by the faster extinction of DEP20 relative to the LiCl group the strength of the aversive memory induced by DEP20 was weaker in comparison to the one produced by LiCl, (RM ANOVA EXT1-3, main effect; $F_{(1, 10)} = 5.41$. $P = 0.042$, across days $F_{(1, 2)} = 30.91$, $P < 0.0001$). In summary, both DEP20 and LiCl reduced the consumption of a novel tastant.

In light of this result, we tested whether DEP20-induced LFP's oscillations could reflect any kind of aversive or gastric malaise induced brain state. Therefore, we recorded in LFPs the NAc shell while the same rat received saline, LiCl and finally DEP20 in the same session. Figure 22B shows the spectrogram of a representative experiment and Figure 22C shows the normalized PSD LFP's changes. It is seen that the injection of LiCl, despite the presence of a transit peak, showed a non-significant decrease in delta and theta oscillations but DEP20 significantly increased beta, theta and delta oscillations (Kruskal-Wallis; $H_{(2)} = 13124$, $P < 0.0001$, $H_{(2)} = 4146$, $P < 0.0001$, $H_{(2)} = 118$, $P < 0.0001$, respectively). The hypnogram is shown below panel 22B. Note that after DEP20, rats are primarily in the active awake state (green) with increased LFP's oscillations, whereas LiCl infusion prevents SWS (gray) by inducing gastric malaise but without increasing the LFP oscillations. The average locomotion (Fig. 22D) revealed that LiCl showed a "spike-patterned" locomotion profile that is likely caused by the distress, and not because LiCl increased exploratory locomotion as does DEP20. In summary, these data clearly shows that

the LFP oscillations in the NAc shell evoked by LiCl, a prototypical gastric malaise agent, and DEP20 are significantly distinct.

7. Discussion

The most common recommendations for persons wanting to lose weight is dietary regimen, exercise and, in many cases, the short term use of appetite suppressant drugs. Many prescribed suppressants, like diethylpropion (DEP), phentermine (PHEN) and d-norpseudoephedrine (NORP), are mild psychostimulants and, as such, increase wakefulness, increase psychomotor activity, cause changes in mood, and produce weight loss (Silverstone 1992). To cause all these changes these compounds must affect many brain areas, in part, by increasing the concentration of neurotransmitters such as serotonin (to reduce craving), norepinephrine (to increase activity and metabolism) and dopamine (for reward and motor activity)(Rothman et al. 2002). Nevertheless, despite their extensive usage, their evoked behavioral changes and their corresponding neuronal correlates remain poorly understood. For this reason, in rats, we investigated behavioral and concurrently neural responses in the nucleus accumbens shell (NAc shell) to repeated systemic treatments of DEP, PHEN and NORP.

In this study we found that all three anorexic compounds increased weight loss, decreased food intake and increased locomotion and stereotypy (less pronounced with NORP). However, the animals developed drug tolerance (tachyphylaxis) to all of these drugs with increasing treatments. Recordings of responses from the NAc shell revealed that all the three appetite suppressant drugs evoked a global inhibitory imbalance (DEP=PHEN>>NORP) involving all putative neuronal types that correlated with the onset of locomotion and stereotypy. Analysis of the LFPs revealed that these compounds initially increased delta, theta and beta oscillations, which correlated with the animal's initial weight loss and decrease in food intake also with the subsequent tolerance developed during treatment. In addition, we identified roles for dopamine D1-like and D2-like receptors in the DEP20-induced effect on weight loss, food intake, locomotion, stereotypy, global inhibition of NAc shell spiking activity and changes in LFP oscillations. Thus,

activation of D1-like and D2-like receptors by these appetite suppressants are important factors in the ability of this class of anorectic compounds to induce weight loss and corresponding behavioral changes.

7.1 Comparison of the appetite suppressants:

Previous studies in humans revealed that at appropriate dosage concentrations DEP, PHEN and NORP produce weight loss (Hendricks et al. 2009) with the order DEP ≥ PHEN > NORP (Cercato et al. 2009; Kutscher 1987; Suplicy et al. 2014). Here, using rats, we found that DEP20 was slightly more potent than PHEN20 with regard to weight loss (Fig. 5), locomotion, and stereotypy (Fig. 7) although for food intake it was equipotent (suppression). Moreover, NORP also found to be equally potent to induce weight-loss and food suppression but different from DEP and PHEN to induce tachyphlaxis and stereotypy. Nevertheless our data show that these three drugs display many similarties. For example, the efficacy of the drugs were not significantly different with respect to times in behavioral states (Table 5), putative cell-types of NAc shell neurons activated or inhibited (Tables 3 and 4), changes in firing rates (Inhibited/Activated; Table 2), and effects on LFPs (Table 6 and Fig. 15). Thus in the NAc shell these appetite suppressant drugs evoke a similar neuronal signature. In this regard, similarities were expected since these drugs are all mild congeners of amphetamine.

The effects of these appetite suppressants drugs on weight loss and food intake are of clinical interest because after the initial decrease of weight and food intake the animals developed tolerance to them which resulted in increasing their food intake (less observed with NORP) and re-gaining weight (Figs. 5 and 8). These results parallel findings in humans taking these same appetite suppressant drugs (Fernstrom and Choi 2008). Although, humans develop tolerance to the weight loss effects induced by appetite suppressants with a different time scale (in months, not weeks), they follow the same pattern as rats. For humans, after 5-6 months on treatment their weight loss plateaus. From this point their body weight is either maintained or is slowly increased (Cercato et al. 2009). Although the mechanisms explaining why appetite suppressant drugs developed tolerance are largely

unknown, here our results suggest that LFP oscillations in the NAc shell can be used as a biomarker to determine when tolerance to DEP20 develops (Fig. 21).

7.2 Locomotion and stereotypy

Locomotion involves any type of forward non-repetitive movement whereas stereotypy behaviors are repetitive motor patterns, such as head oscillations, with no obvious function (Mason 1991). Although it has been known that DEP induces locomotor activity in rodents (Reimer et al. 1995), we found DEP10 produced the greater locomotion but DEP80 decreased its locomotion which reflects the impairment of the locomotor activity accompanied with the stereotypic behaviors [Fig. 7A; (Safta et al. 1976)]. A result similar to that observed after chronic treatment with amphetamine (Borison et al. 1977). Moreover, like DEP and amphetamine (Safta et al. 1976), PHEN20 and NORP20 also induce head-weaving stereotypy (Fig. 7B and C), suggesting this behavior is a hallmark of this class of appetite suppressant drugs. The rapid onset of locomotor activity suggests that DEP can be rapidly metabolized and cross the blood brain barrier (Carlsson and Johansson 1978; Cox and Maickel 1972).

7.3 Behavioral mechanisms of appetite suppressant drugs anorexia

In our study we used information that showed that amphetamine delayed the onset of eating and increased the inter-meal interval (Blundell et al. 1976). These data rule out an effect on satiety signals, but support the idea that amphetamine-induced anorexia evokes motor stimulatory effects that compete (interfere) with eating (Wolgin 2000). In agreement with this idea, our data indicate that, rats treated with appetite suppressant drugs completely neglect chow food during the initial 2 h after treatment (Ghosh and Parvathy 1976). In other words, the data suggest that the greater the locomotion and stereotypy, the greater the food suppression on appetitive behaviors.

Another mechanism of appetite-suppressant drugs to induce anorexia is its potential to induce gastric malaise. It is known that amphetamine can induce conditioned taste aversion (CTA) [see Carey (1973)]. Here we show that DEP20, and presumably like other appetite suppressant drugs, can also induce CTA to a

novel palatable taste (Fenu and Di Chiara 2003). We suggest that DEP20's ability to induce CTA can partially contribute to its anorectic effects by decreasing the consumption of novel foods and/or by reducing the probability of trying novel foods. However, DEP20's anorectic effect induced on a highly familiar food cannot readily be attributed to CTA because it is much more difficult to acquire aversion to familiar foods (Garcia et al. 1974). Moreover, because the animals increased their food intake across injection days (Figs. 5A bottom panels and 21A), it is unlikely this can be attributed to conditioned taste aversion, which shows little tolerance during extinction (Garcia et al. 1974). Thus, not all anorectic effects of appetite suppressant drugs can be accounted by its potential to induce gastric malaise.

In summary, the behavioral effects induced by these appetite suppressants reflect the strong interconnection among different brain networks controlling weight-loss, eating, sleep, locomotion and stereotypy (Costa et al. 2007; Nicola 2010; Seiden et al. 1993; Tellez et al. 2012). As outlined below, dopamine plays important roles in relating all these behaviors (de Araujo et al. 2012; Dzirasa et al. 2006).

7.4 Neuronal modulation of the NAc shell single-unit activity

To determine possible neuronal correlates underlying these behavioral effects we performed recordings in the NAc shell, a brain region that integrates inputs from limbic brain regions and transmits them to motor and eating regions (Hajnal and Norgren 2004; Maeda and Mogenson 1980). Although appetite suppressant drugs are likely acting in multiple brain regions and that the NAc shell is not the only area important for the action of these appetite suppressants (Shi et al. 2000). Here we found that DA receptors in the NAc shell contribute, to most if not all, of the pharmacological effects induced by amphetamine related appetite suppressant drugs (Fig. 16). Furthermore, the NAc shell is a critical region for amphetamine's effects since direct infusions (See Fig. 4) of large doses of amphetamine into the NAc shell can suppress food intake and stimulate locomotion (Carr and White 1986; Kelley et al. 1989). In contrast, lower doses of amphetamine can promote food intake (Evans and Vaccarino 1990; 1986; Wise et al. 1989), suggesting that the level of dopamine stimulation in the NAc can have a bidirectional control upon food intake.

In the same vein, it has been shown that pharmacological inactivation of the NAc shell using low doses of the GABA agonist muscimol promotes eating behaviors, although higher doses suppressed food intake, and induced locomotion (Kelley et al. 1989; Stratford and Kelley 1997).

This bimodal effect has been substantiated by the finding of a moderate inhibitory imbalance in the population NAc shell produced by rats eating hedonically positive foods (Krause et al. 2010; Tellez et al. 2012), whereas Krause et al. 2010 showed that electrical stimulation of the NAc shell stops eating, uncovering that NAc activity can have a bidirectional control upon eating behaviors. Here we found a large inhibitory unbalanced in the rat NAc activity obtained with appetite suppressant drugs, which decreases food intake and increases locomotion and stereotypy. We are aware that these data add a degree of complexity to both the "gating feeding hypothesis" which states that inhibition in the NAc allows ingestive behavior (Krause et al. 2010) and to the "NAc decreased (reward) / increased (aversion) activity hypothesis" (Carlezon and Thomas 2009). Thus, the strength of the inhibitory imbalance of the population NAc shell activity can correlate with multiple and sometimes conflicting behavioral outputs, such as food intake and appetite suppressant's induced-anorexia.

An important result of this study was that all three amphetamine related appetite suppressant drugs evoked a large inhibitory imbalance in the NAc shell (although NORP was not stronger but showed a trend) that involved all putative cell types and that matched the onset and continuance of locomotion and stereotypy (Fig. 21 and Table 4). These locomotor behaviors are incompatible both with food intake and sleep. These data also show that pSPNs are the neurons most likely to be activated by DEP20 (Table 4). In summary, we have identified a correlation between changes in NAc shell single unit activity and the onset of motor changes.

7.5 LFP's oscillations in the NAc shell

Whereas appetite suppressant drugs cause a rapid imbalance in the firing rate of NAc shell neurons that correlate with the onset of locomotion and stereotypy, changes in LFPs correlate with the longer-term behaviors involving food intake and

the accompanying weight loss. This behavior is illustrated in experiments with DEP20 over 14 days (Fig. 21 and Table 7). However, for DEP20 one day after treatment beta, theta and delta oscillations were large and positive and that this change is associated with the largest decrease in food intake (Fig. 21). However, on subsequent treatment days the magnitude of the three oscillations decreased and food intake increased. This is the first report regarding the measurement of the temporal changes evoked by appetite suppressant drugs in the NAc shell and their relation to food intake. We are aware that these oscillations are also involved in motor and other behaviors (Jenkinson and Brown 2011) and that in rats cortical EEG's studies that have shown that DEP evokes robust cortical delta oscillations (Safta et al. 1976). We hypothesize that amplification of delta oscillations during awake states, over those seen in SWS (Steriade et al. 2001) is related to conditions were DA levels are out of their physiological dynamic range (Costa et al. 2006; Lemaire et al. 2012) and under situations where energy consumption needs to be reduced (Dworak et al. 2011). Likewise, beta oscillations are normally high during tonic muscle rigidity in preparation to voluntary movement, but they are amplified in Parkinson disease patients where DA levels are lower of its physiological range (Jenkinson and Brown 2011). It is possible that the decrease of DEP20- induced beta oscillations might be related to a DA alteration after chronic use, whereas theta oscillations induced by appetite suppressant drugs might be related to locomotion and exploratory head weaving behaviors (Buzsaki 2005).

7.6 Dopamine receptors and DEP's effect

The three amphetamine related appetite suppressants tested induce the release of norepinephrine, serotonin and dopamine (Opacka-Juffry et al. 2014). Here we show that their appetite suppressing and motor effects, as well as changes in NAc shell activity, can in part be accounted for by their ability to release dopamine. In this regard, amphetamine also causes DA to be released in the rat NAc (Daberkow et al. 2013) and also alters their eating and motor behaviors (stereotypies)(Seiden et al. 1993). Indeed, a general dopaminergic tone appears to be necessary to express the full magnitude of locomotor activity and stereotypy induced by

amphetamine (Sotnikova et al. 2005), DEP (Samanin and Garattini 1993), PHEN (Offermeier and Potgieter 1972). What has not been tested is how these drugs affect the NAc shell activity and if DA antagonists could reverse their effects. Here we showed all the three appetite suppressant drugs effects on locomotion was attenuated by intraNAc shell infusions of D1R and D2R antagonists (Fig. 17). In agreement with the proposed role of these receptors, we found that SCH23390 strongly delays the onset and magnitude of locomotion and stereotypy suggesting that activation of D1Rs are necessary for triggering these behaviors (Szczypka et al. 1999). Our data also indicates that D1Rs can also be involved in food intake and weight loss. In agreement with D2Rs prominent role mediating energy homeostasis (Kim et al. 2010) and obesity (Johnson and Kenny 2010), we observed that D2R antagonist reversed the anorectic and weight loss effects induced by appetite suppressant drugs and (when infused i.g.) reverse the net inhibitory imbalance in the NAc shell produced by DEP. Nevertheless, D2Rs seem to also play an important role triggering locomotion and stereotypy. Our data favors the idea of a more cooperative action of both D1 and D2 like receptors -in the brain and in the NAc- mediating the pharmacological effects of this class of appetite suppressant drugs.

8. Conclusions

In summary, these results demonstrate that amphetamine related appetite suppressant drugs modulate weight loss, food intake, locomotion and stereotypy. Finally, in contrast to the belief that serotonin and norepinephrine are major contributors to the action of these drugs, we found that both their induced behavioral and electrophysiological changes were largely reversed by D1R and D2R antagonists, thus providing a role for these DA receptors in the anorexia induced by these appetite suppressant drugs.

9. Perspectives

Currently, pharmacological obesity treatment options are limited. However, new antiobesity drugs acting through central nervous system pathways or the peripheral adiposity signals and gastrointestinal tract are under clinical development. One of the most promising approaches is considering that any anti-obesity therapy may affect one or several of the systems that control food intake and energy expenditure. However, our results demonstrated the neuronal mechanism of this class of appetite suppressant drugs that has been approved and commercially used for more than 60 years for the treatment of obesity. Activation of dopamine receptors of the rat's brain reward system (NAc shell) by this kind of appetite suppressant drugs further support the idea towards the potential use of this pharmacologic therapy for the treatment of obesity. Progress in this area might perhaps, showed a possibility to develop new effective drugs without the central stimulant effects or addiction potential and to identify and develop anorectic agent acting specifically on ingestive behavior, potentially acting specifically on particular dopamine receptor. In recent years the use of peptides that influence the peripheral satiety signals and gut-brain axis such as glucagon like peptide (GLP-1) analogs are also under development. However, it is unlikely that a single pharmacological agent will be effective as a striking obesity treatment. Thus, future strategies to treat obesity will need to be directed at sustainable weight loss to ensure maximal safety. This strategy will probably require the co-administration of medications that act through different mechanisms.

10. PUBLICATIONS

Kalyanasundar, B., C. I. Perez, A. Luna, J. Solorio, M. G. Moreno, D. Elias, S. A. Simon, and R. Gutierrez. "D1 and D2 antagonists reverse the effects of appetite suppressants on weight loss, food intake, locomotion and rebalance spiking inhibition in the rat NAc shell." Journal of neurophysiology (2015): Vol. 114 no. 1, 585-607 DOI: 10.1152/jn.00012.2015.

Kalyanasundar B, Jessica Solorio, Claudia I. Perez, Sidney A. Simon and Ranier Gutierrez. *The efficacy of the appetite suppressant, diethylpropion, is dependent on both when it is given (day vs. night) and under conditions of high fat dietary restriction***.** Appetite. (In revision).

Kalyanasundar B, Moreno MG , Sidney A. Simon and Ranier Gutierrez. *The appetite suppressant D-norpseudoephedrine (Cathine) modulates the spiking activity of the nucleus accumbens shell and acts via activation of D1/D2 dopamine receptors*. Front.Pharmacol- under submission process.

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