The endocannabinoid system as possible target for the treatment of obesity related disorders: beyond cannabinoid receptors

A Dissertation in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Pharmacology and Toxicology

Marialuisa de Ceglia

Department of Physiology and Pharmacology "Vittorio Erspamer"
Sapienza University of Rome

Director of PhD Program:
Prof. Silvana Gaetani

Thesis Advisor:
Prof. Silvana Gaetani

Thesis Committee:
Prof. Luìs Javier Santìn Nuñez
Prof. Giuseppe Battaglia
Prof. Carlo Polidori

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CHAPTER 1 – INTRODUCTION

1. OBESITY

At this moment, the number of obese people in the world is supposed to be around 2.1 billion; which represent 30% of total adult population[1] and, unfortunately, this number rises every day. It was in the 1990s when the World Health Organization began sounding the alarm of obesity being a new pandemic disease[2]. In the same years, public awareness campaigns were initiated to sensitize policy-makers, private sector partners, medical professionals and the public at large; but they did not result in an effective action in slowing the diffusion of the pathology. Over time, obesity epidemic diffused not only in industrialized societies; but also in developing countries; thus transforming it in a world-wide concern[2].

Obesity is generally characterized by excessive accumulation of body fat in various districts of the body. Clinically, the most used parameter to define overweight and obesity is the body mass index or BMI, which is obtained by dividing a person's weight in kilograms by his squared height in meters. The value obtained defines different classes of pathological conditions, such as:

- BMI < 16,5: severely underweight
- 16,5 < BMI > 18,4: underweight
- 18,5 < BMI > 24,9: normal weight
- 25 < BMI > 29,9: overweight
- 30 < BMI > 34,9: obese I class (moderate obesity)
- 35 < BMI > 39,8: obese II class (severe obesity)
- BMI > 40: obese III class (very severely obese)

BMI is the most used index to describe body mass and obesity; but it doesn't take into account lots of other factors; such as gender, age, percentage of lean/fat mass[3]. For this reason, it is always accompanied by other measures, for example the

abdominal circumference, since the accumulation of fat in the visceral area is correlated to cardiovascular and metabolic disorders[4].

Lots of factors can influence the development of obesity: there appears to be a complex relationship among biological, psychosocial, and behavioural factors, which include genetic makeup, socioeconomic status, and cultural influence[5]. Independently from the clinical reason which causes obesity development, hyperphagia or chronic overeating is a primary behavioural component of this pathology[6,7]; many forms of obesity can be considered as a consequence of overeating[8]. This deregulated feeding behaviour results from increased availability of calorie-dense foods[8,9], reduction of physical activity due to a sedentary lifestyle, unhealthy eating habits, increased stress exposure[10,11], which contributed to the mass diffusion of the pathology[12].

Besides environmental factors, obesity development has been linked to microorganisms, epigenetics, increased maternal age, lack of sleep, endocrine disruptors, pharmaceutical iatrogenesis; comorbid conditions and their treatments may also be an important factor[13]. Table 1 gives an insight into the most common causes of obesity.

Primary causes of obesity	Secondary causes of obesity
Genetic causes	**Neurologic**
	Brain injury
Monogenetic disorders:	Brain tumor
Melanocortin-4 receptor mutation	Consequences of cranial irradiation
Leptin deficiency	Hypothalamic obesity
Proopiomelanocortin deficiency	
	Endocrine
Syndromes	Hypothyroidism
Prader Willy	Cushing syndrome
Bardet Biedl	Growth ormone deficiency
Cohen	Pseudohypoparathyroidism
Alstrom	
Froehlich	**Psycological**
	Depression
	Eating disorders
	Drug-induced
	Tricyclic antidepressants, oral contraceptives, antipsychotics,anticonvulsants, glucocorticoids, sulfonylureas, glitazones, beta blockers

Table 1: Summary of the principal causes of obesity. The table was extracted from [14].

For this reason, we can state that obesity is a heterogeneous disorder[15]. In the last years, as new mechanisms behind obesity development were discovered, researchers differentiated obesity into two different types: metabolic and hedonic[15,16]. In particular, metabolic obesity is associated with impairments of the homeostatic control of food intake whereas hedonic obesity is characterized by disruptions of the reward system[16] that cause persistent overeating, increased ratio between energy expenditure and body mass, comorbidity with eating disorders, depression, anxiety, stress, sleep deprivation[15].

Alongside with its spread diffusion, what makes obesity an important pathology to study is its harmfulness: for both men and women, it has been observed a progressive increase of the risk of death with an increased degree of obesity[17]. In fact, obesity is considered a risk factor for the development of numerous pathologies such as:

- Cardiovascular diseases: hypertension[18], myocardial infarction[19], stroke[20];
- Metabolic disorders, such as type 2 diabetes[21], chronic hyperglycemia[22];
- Dementia[23], Alzheimer's' disease[24];
- Cancer, especially breast and endometrium one[17]; colon and kidney[25];
- Hepatic dysfunction and cirrhosis[17];
- Pulmonary diseases such as sleep apnea and asthma[26];
- Osteoarthritis[27];
- Fertility, pregnancy and delivery complications[28];
- Increase of anxiety, depression and suicide[29].

Up to date, several aspects regarding obesity are still unknown; among them, the availability of effective treatments is the most urgent. The majority of the efforts in containing the pathology are based on prevention: individuals have to limit energy intake from fats and sugars, increase the consumption of fruit and vegetables, have a healthy and active lifestyle with regular physical activity[2]. When prevention is not sufficient and obesity is developed, the first type of intervention focuses on diet control and physical exercise, accompanied by various behavioural therapy techniques, such as self-monitoring, goal-setting, cognitive restructuring,

mindfulness[30]. This type of behavioural intervention often fails in producing sustained weight loss and so it is accompanied by pharmacological or surgical treatments[31], which can be used alone or in combination[32]. FDA approved pharmacological treatments available up-to date include: the 5-HT2c receptor agonist lorcaserin (Belviq); glucagon-like peptide-1 receptor (GLP-1R) agonist liraglutide (Saxenda); or complex polypharmacological combination of phentermine/topiramate in extended release (Qsymia) and naltrexone/bupropion (Contrave)[33]. Among all these available treatments, only liraglutide and naltrexone/bupropion combinations have been approved as weight loss agents also by EMA[31]. Surgical treatments include: gastric bypass, vertical sleeve gastrectomy, sclerotherapy, intragastric balloon, biliopancreatic diversion[34]. Therefore, no one-size-fit-all treatment has been encountered[35], since treatments not only have to promote weight loss and body fat reduction, but also have to change the way brain responds to food stimuli[36].

2. PHYSIOLOGICAL CONTROL OF FOOD INTAKE

The fundamental cause of obesity and overweight is an energy imbalance between caloric intake and energy expenditure (basal metabolism, body temperature maintenance, physical activity): when energy intake exceeds the energy expenditure, the excessive energy can be stored as fat, bringing to the development of obesity[37]. Energy balance is controlled by multiple physiological signals, which go from the periphery (where adipose tissue acts as storage and liver controls lipid and glucose metabolism[38]) to the central nervous system (CNS; that acts as an integration centre and elaborates behavioural responses[39]) and vice versa.

There are numerous mechanisms by which the CNS controls food intake and receives information about it, and they involve both neural control of the gut and chemicals (hormones and peptides) which act as signals to regulate feeding.

In particular, we can talk about the gut-brain axis: gastrointestinal tract (GI) is innervated by the autonomic nervous system (parasympathetic and sympathetic divisions). Most important nerves are: the vagus and pelvic nerves, which exert an inhibitory effect on GI and the splanchnic nerves, which exert an excitatory effect. In

addition to these components, there is also an enteric nervous system (ENS), composed by a set of neurons located in the myenteric plexus[40]. The ENS is controlled by the CNS thanks to vagal and pelvic efferents. These fibres convey to the CNS all the stimuli produced in the intestine, both mechanical or chemical, such as distension, contraction, neurotransmitters, nutrients, cytokines, mediators of inflammation, hormones[41]. On the other hand, splanchnic efferent neurons regulate nociception and stress response[42].

In addition to all these nerves which control in-situ the GI tract, there are several regions of the brain that are involved in the control of food intake; in particular circumventricular organs lacking a functional blood-brain barrier (BBB), such as the area postrema (AP)[43], which is activated by levels of circulating nutrients and, in turn, activates other nuclei in the brainstem[44]. Brainstem nuclei, such as the nucleus of the solitary tract (NST), are involved in the control of vagal efferents and modulation of the ENS and, at the same time, receive information from the periphery[45] and project to hypothalamic nuclei[45,46]. Hypothalamus communicates with vagal efferents[9] and regulates appetite through various molecules and receptors, such as endocannabinoids[47], neuropeptide Y (NPY), pro-opiomelanocortin (POMC), alpha-melanocyte-stimulating hormone (α-MSH), Agouti-related peptide (AgRP), cocaine- and amphetamine-regulated transcript (CART), cholecystokinin (CCK), and glucagon-like peptide 1 (GLP-1)[32,48].

As said before, food intake is modulated by a lot of peptide and hormonal mediators, released both by periphery and CNS; these signals regulate feeding behaviour, food processing, intestinal motility and metabolism.

The GI, in particular, produces more than 20 different mediators[49]. Gastric distension and the presence of nutrients in the intestine activate the release of peptide YY (PYY), GLP-1, oxyntomodulin (OXM); which act on endocrine glands, smooth muscle and peripheral nervous system[50,51]. Other hormones play crucial roles in the sensation of hunger and satiety; for example, CCK, PYY, GLP-1 and OXM can reduce food intake by acting on the orexigenic and anorexigenic signals in the hypothalamus[52,53] and at the same time enhance the feeling of satiety during the interval between meals regulating GI motility[54,55].

Talking about hormones, the peptide ghrelin is produced by the stomach and released into the bloodstream. Ghrelin is made up by 28 amino acids and undergoes post-transcriptional addition of octanoic acid on the serine 3 [56] which enables it to cross the BBB and bind the growth hormone secretagogue receptor (GHS-R)[57]. Levels of ghrelin increase during fasting and decrease after a meal; for this reason, it is defined as the "hunger hormone"[57]. Peripheral administration of ghrelin reduces the use of fat from adipose storage; whereas the central administration of ghrelin produces an increase of food intake and release of growth hormone in rats[58]. Disruption of ghrelin signalling causes alterations in energy homeostasis[59,60]: pharmacological inhibition of GHS-R was thought as a strategy in the treatment of obesity but even if GHS-R antagonists were able to decrease food intake in fasted animals[60] and a vaccine against ghrelin induced weight loss in preclinical studies[61], no promising results were obtained for human treatment of obesity[56]. On the other hand, agonists of ghrelin were used for the treatment of anorexia in patients experiencing appetite loss or malnutrition[62].

PYY is a peptide hormone related to NPY. Both peptides bind Y receptors and have a characteristic structure deriving from the presence of PP proteins. In our bodies, PYY is present in two different isoforms, which differ for the presence of the N-terminal, PYY_{1-36} and PYY_{3-36}. Numerous studies suggest that only the last one has a higher affinity towards the Y2 receptor (Y2R)[53]. Acute administration of PYY_{3-36} can reduce food intake[63].

Among all the hormones that regulate food intake, another important one is CKK. CCK was the first intestinal hormone to be identified as involved in the regulation of feeding behaviour[64]. Produced in the duodenum, it is released during the after-meal period and acts thanks to the activation of CCK receptor 1 (CCK1) in vagal fibres. Antagonists of CCK1 increase food intake in rodents and humans[56,65]; while KO animals for CCK1 are obese and hyperfagic[65]. Despite this, continuous infusions of CCK are unable to reduce food intake[66].

Pancreatic polypeptide (PP) is synthetized in the endocrine pancreatic parenchyma and has high affinity for Y4 and Y5 receptors. Its levels increase after a meal and decrease during fasting[67]. Acute administration of PP reduces food intake in mice and humans[67,68]; while chronic administration reduces body weight in ob/ob mice[69]. Recent studies assume that PP is able to delay gastric emptying, carrying out an anorexiant effect[70].

Another peptide involved in the control of food intake is amylin; released by pancreatic beta cells after the ingestion of food. The 37 aminoacidic peptide has a role in glucose homeostasis and is able to reduce food intake after peripheral administration[71]. It seems that modulation of noradrenergic, histaminergic and dopaminergic systems is involved in the anorexigenic effect of amylin[72]. Moreover, it has been reported that obese patients present an increase of the circulating levels of amylin[73]. An analogue of amylin, Pramlintide, is used to reduce body weight in patients with type 1 and 2 diabetes[74,75].

An important precursor of numerous hormones influencing food intake is preproglucagon. In L cells of intestinal mucosa, preproglucagon is cleaved into glucagon, GLP-1, GLP-2, OXM[76].

Glucagon is produced by α-cells of Langerhans islets, in hypoglycaemic states; its release increases energy expenditure[77] and reduces food intake by modulating vagal tone and gastric emptying[78].

GLP-1 presents two different active forms for CNS and periphery. In the CNS GLP-1 positive neurons are present in paraventricular nucleus (PVN), dorsomedial nucleus (DMN), hypophysis and thalamus[79]. GLP-1 is considered a potent incretin: it is released upon food intake and stimulates the release of insulin[56]. GLP-1 peripheral administration reduces appetite in both humans and rodents[80]. Agonist of GLP-1 exendin is used in a phase 3 clinical trial to ameliorate glucose homeostasis and reduce food intake in type 2 diabetes patients. Moreover, GLP-1R agonist exenatide is already approved as a co-treatment for type 2 diabetes[81].

GLP-2 has been found in high concentrations in the brain; where its administration reduces food intake[82]. Peripherally, GLP-2 is able to regulate gastric motility, digestion and absorption of nutrients; without affecting appetite[80].
Also OXM is released upon the ingestion of food and is able to reduce food intake when administered centrally[83].

Another important hormone related with food intake and glucose homeostasis is insulin. It is produced by pancreas beta-cells and released upon food ingestion. Together with its well-known hypoglycaemic effect, insulin acts in the CNS as a satiety factor[84]: insulin receptors are expressed in various regions of the brain, such as arcuate (ARC), DMN and PVN[85].

Alongside with all these peptide mediators produced in periphery, other molecules regulating food intake are produced in the brain.
One of the most important orexigenic neuropeptides is NPY; which is synthetized by neurons in ARC and released in the PVN. Synthesis and release of NPY are regulated by leptin, insulin and ghrelin[86]. Different receptor of NPY have been discovered (Y1, Y2, Y3, Y4, Y5, Y6); among them Y5 receptor seems to be the most involved in the modulation of feeding behaviour[87]. Central administration of NPY is able to increase appetite[86].

Another peptide, produced in ARC and with orexigenic effect as NPY, is AgRP. Central administration of AgRP in PVN or DMN results in increased food intake[88].

Other orexigenic peptides are the hypocretins 1 and 2; mostly known as orexin A and B. Differently from AgRP and NPY, these peptides are produced in the lateral hypothalamus and stimulate appetite thanks to the activation of orexin receptors OX1R and OX2R; mainly expressed in ventromedial hypothalamus and PVN respectively[89]. Of course, neurons secreting orexins project also to other hypothalamic nuclei; for example in the ARC there are some NPY secreting neurons expressing OX1R; thus demonstrating that these peptides work in synergy[90].

In addition to orexigenic signals, the brain produces also anorexigenic ones: between them we can find melanocortin, deriving from post-transcriptional cleavage of POMC, whose gene is expressed throughout the hypothalamus, adenohypophysis, pars intermedia[91]. Another melanocortin, α-MSH, is produced in the hypophysis and activates melanocortin receptors, expressed in brain areas linked to the control of feeding behaviour, such as the cortex[91].

Other important anorexigenic signal in the brain is CART, which is synthetized in the hypothalamus, and regulates many processes, including food intake, maintenance of body weight, reward and endocrine functions through activation of CART receptors, which are expressed both in the brain and in the gut[92].

Lastly, other neuropeptide involved in the control of food intake is the corticotropin-releasing hormone (CRH), highly expressed in the PVN. This 41 aminoacidic peptide is able, when centrally administered, to inhibit food intake and body weight in rats[93]. In humans, peripheral administration of CRH is able to increase energy expenditure and fatty acid oxidation[93].

As I will further explain in other paragraphs of this thesis, endocannabinoids, lipid mediators and neurotransmitters also can regulate food intake, nutrition, body weight. Between them, also histamine can be considered an hypophagic agent. Histamine is produced by decarboxylation of histidine by the enzyme histidine decarboxylase[94]. Histaminergic system is involved in the anticipatory phase of eating and in the consummatory phase of feeding behaviour[95]. Appetite-suppressing action of histamine is linked to its effect on H1R in ventromedial hypothalamus[96]; that's why intra-hypothalamic administration of histamine induces satiety[97].

3. FOOD ADDICTION

As said before, one of the biggest problems in obesity is overeating. But why do people eat much more than what they need for their energy expenditure? Even if this seems a simple question, no clear answer is available up-to-date. Since 1950 more

and more studies began comparing obesity and drug addiction, since they are both characterized by repeated behaviours that the individual is unable to control despite awareness of undesirable consequences[98]. In 1956, Randolph first introduced the concept of food addiction, referring to a common pattern of symptoms similar to those of other addictive diseases observed in the consumption of foods such as corn, wheat, coffee, milk, eggs and potatoes[36].

There are several similarities between addicted and obese persons: obese people tend to be more heavily influenced by immediate rewards and less responsive to future consequences, as drug addicted individuals[99]. Moreover, impulsivity and inhibitory control is impaired both in overeating and drug addiction[100]. Obese individuals have enhanced attentional processing of food and food-related stimuli, same as individuals with addiction to cocaine, opiates, alcohol, nicotine, cannabis and caffeine with drug-related stimuli[101]. In addition, people with higher BMI score higher on neuroticism and extraversion and lower on conscientiousness, personality traits typical of drug-addicted individuals[101].

However, the possibility for food to be as addictive as drugs is still largely debated, since food addiction continues to evolve in an extremely complex and multidimensional pathology[102]. Up to date, food addiction has not been listed in the Diagnostic and Statistical manual of Mental Disorders (DMS V)[103] and the concept still appears controversial, considering that feeding, unlike drug self-administration, is a natural reward necessary to survival[8,104]. Moreover, obesity is a heterogenous and multifactorial pathology that can depend on lifestyle or other hormonal, genetic conditions; differently from drug addiction[101]. Unlike addictive drugs, it's difficult to distinguish from normal consumption and compulsive abuse. Furthermore, the addictive component of food has yet to be clarified[105]. Some evidence suggests that combination of macronutrients is an important determinant of compulsive overeating: modern highly processed foods rich in fat and refined sugars are the one involved in problematic addictive-like behaviours[106]; in fact cafeteria-style diet exposure (characterized by consumption of highly processed and high-palatable food, rich in sugar and fat) tends to increase overall feeding frequency, food approach behaviour, energy intake and weight gain[8].

In this panorama of increased obesity rates around the world and increased tendency to consume highly processed foods to overcome negative states deriving from the mind[8], researchers tried to develop a more precise way to define food addiction, and that's when Gearhardt et al. developed the Yale Food Addiction Scale (YFAS)[107]; composed by selected questions and used to assess food addiction diagnosis in a large number of studies[108]. Other markers used to study food addiction are the reward based eating scale[109] or nausea and increased salivary cortisol elicited by naltrexone[110].

Besides defining and measuring food addiction, another important focus of reaserch has been investigating the mechanisms at the base of it: what are the neural circuits involved in hedonic eating and what the variations that need to occur to cause addiction?

First of all, it is important to notice that eating is intrinsically rewarding and that the consumption of palatable food is able to activate the reward system: the neural circuitry responsible for regulating homeostatic and hedonic feeding are heavily integrated, regulate feeding behaviour interindipendently[111,112] and usually harmonize their actions well; since in a physiological state metabolic signals and the pleasure of eating are coordinated to drive food consumption[15]. Foods high in fat and sugar impair both the metabolic and the reward system: the development of metabolic or hedonic obesity is linked to different sets of susceptibility genes[15]. In people addicted to food, overeating may not cause leptin resistance for example, but changes in the reward system that lead to uncontrollable desire of food and excessive food intake[113,114]: obese people in fact show higher responsivity in reward and attention regions of the brain in response to food cues[36].

As any other addiction, food addiction is characterized by three salient phases which allow to distinguish occasional and controlled use from the chronic dependence: binge/intoxication; withdrawal/negative affect and preoccupation/anticipation (craving)[36].

Bingeing stage involves nucleus accumbens (ACC) and dorsal striatum (DLS); the withdrawal stage is due to the activation of amygdala (AMY), stria terminalis, ACC; whereas the craving stage is due to prefrontal cortex (PFC), hippocampus and insula

activation[36].

In all these areas the main impairments involve the dopaminergic, opioid and endocannabinoid systems, which are strictly interconnected. The role of these systems in food addiction has been confirmed by preclinical and clinical results of different psychoactive medications aimed at controlling food consumption[36]. In particular, opioid system is associated with pleasure deriving from food reward and it acts synergically with the dopaminergic one in promoting food intake[115]. Opioid and endocannabinoids modulate functions of palatability, that is "liking" of food[15] and cause delayed increase of dopamine (DA) (which mediates "wanting" of food) as a function of increased glucose and insulin[98]. As a matter of fact, changes in insulin signalling in reward regions result in disrupted DA homeostasis[116]: several studies have demonstrated that impaired glucose metabolism in prefrontal areas of the brain is accompanied in obese people by poor DA signalling in DLS[36]. DA deficiency occurs with low striatal availability of D2 (dopamine receptor type 2) in obese people[117], that may put these individuals at higher risk of compulsive eating, due to impaired ability of DA to modulate signalling between DLS and prefrontal regions, which have a role in inhibitory control of food intake[118]. At the same time, lower striatal D2 expression has been linked to augmented glucose metabolism in post-central gyrus and parietal cortex, increased palatability and taste perception, which increment the reinforcing properties of food[119].

Besides DA, also noradrenaline (NA) transmission seems to be altered in obese people suffering FA: fMRI images showed a decrease of NA transporters availability in thalamus[120]; which is important considering that dysfunction of central NA is linked to cognitive and mood disorders and stress responses, including overeating[36]. Differently from the other monoamines, the role of 5HT in food addiction has still to be clarified. For instance, also other mediators may have a role in FA: Cottone et al. discovered alterations to CRF expression in AMY of rats after high-palatable diet withdrawal[121].

It is not excluded that other areas, different from the ones cited before, could be involved in food addiction development: restricted and unrestricted access to cafeteria-style diet for example reduced cannabinoid receptor 1 (CB1) and mu-opioid receptor expression in ventral tegmental area (VTA)[122]. Moreover, seen its role in

addiction, in the modulation of the dopaminergic system and in reward, it's not excluded that food addiction involves several variations to the endocannabinoid system, that have to be investigated[123].

Still lots of aspects related to food addiction remain unclear; however the discovery of the neurobiological and cognitive changes that drive this dependence could result in more targeted and effective psychological and medical treatments of obesity[124,125], that could be treated as a pathology reducing social stigma of hyperphagic obese individuals[101]. At the same time, it will increase public health policies that aim to reduce the consumption of high-palatable food (for example regulating sales, availability, advertising of these foods[101]) in a similar way to what has been done with cigarette smoking[126].

4. OBESITY RELATED DISORDERS: ANXIETY AND DEPRESSION

Anxiety and depression are among the most diffused psychiatric disorders. These two pathologies can co-exist with obesity: for this reason, a lot of researchers have been investigating whether there could be a link between obesity, anxiety and depression.

4.1 ANXIETY

Talking about anxiety, it's important to underline that there are different anxiety disorders, and all of them are characterized by excessive fear of perceived threats in the environment. It's common, in the adult life, to experience fear or anxiety in periods of stress; however, to be diagnosed with an anxiety disorder these symptoms have to persist at least six months. Anxiety can be associated with depression, drug or other substance abuse and withdrawal, clinical conditions such as hyperthyroidism, respiratory disease and cardiac disease. Statistical analysis suggests that at least one person in fourteen has ever experienced an anxiety-like disorder in lifetime[127]. Pathophysiology of anxiety is still poorly understood: studies suggest an over-activation of limbic regions, such as AMY and insula while processing emotional stimuli and aberrant connectivity between these regions and other areas, such as the

prefrontal cortex. Also, some genetic causes have been underlined, and in particular susceptibility in the locus CAMKMT and single-nucleotide polymorphisms on gene MAG11[127].

In the last years, the neuronal circuits involved in the development of anxiety have been the focus of various researches: one of the most important regions playing a role in the pathology is the bed nucleus of the stria terminalis (BNST). This nucleus is part of the extended AMY and receives various projections both from central and basolateral amygdala (CEA, BLA). Along with BNST role with anxiety, studies showed that optical inhibition of cell somata in this region turns in an anxiolytic effect; whereas inhibition of BLA fibres projecting to BNST results in anxiogenic-like behaviour. Conversely, activation of BLA-BNST projections leads to anxiolytic behaviours. Also activation of BNST fibres to hypothalamus has anxiolytic-like effects; whereas projections to parabrachial nucleus lead to anxiogenic-like response. Still it's not clear the role of both GABAergic and glutamatergic fibres in BNST and how their activation/inhibition can mediate anxiolysis[128]. Another important region in the regulation of anxiety is amygdala: somatic activation of BLA projection neurons leads to anxiety-like behaviours; activation of the projections that terminate in CEA has an anxiolytic action; in addition, changes in GABAergic signalling in the amygdala also affects anxiety. Moreover, projections from CEA to ventral hippocampus and medial PFC have been linked to anxiety-like behaviour[128].

Impairments in the monoaminergic systems can have a role in anxiety: talking about DA for example, DA receptor deficiency in prefrontal cortex may fail to inhibit signalling to amygdala with anxiogenic-like response; dopaminergic activity in DLS can affect insular activity, that predicts anxiety symptom's severity[129]. Moreover, patients with social anxiety disorder present DA transporter (DAT) decreased expression and reduced receptor binding in the striatum, compared to healthy controls[130]. Patients with social fear also display lower levels of DA metabolite homovanillic acid (HVA) in the cerebrospinal fluid[131]. On the other hand, symptoms of anxiety also have been linked with hyperactivity of NA in the CNS: in fact, in stress conditions, corticotropin releasing factor can activate NA release in locus coeruleus

(LC) and hippocampus, giving wakefulness and anxiety manifestations. Elevated serum concentration of NA has been linked with anxiety and so was a single-nucleotide polymorphism of adrenergic receptors[129]. Differently from DA and NA, more evidence of 5HT involvement in anxiety has been found: 5HT receptor (5HTR) $5HTR_{1A}$ activation in hippocampus can exert anxiolytic effects; whereas $5HTR_{1A}$ knockout, its mutation or overexpression, and low 5HT neurotransmission have been linked to anxiety-like behaviour in mice. Human studies revealed decreased $5HTR_{1A}$ expression and function in insula, AMY, medial PFC, and raphe nucleus in patients with panic disorder[129]. At the same time, $5HTR_{2A}$ agonism in AMY can cause anxiety; whereas its antagonism has been indicated as anxiolytic in preclinical studies[132]. Furthermore, acute stimulation of $5HTR_{2A}$ and $5HTR_{2C}$ projecting from DR to AMY and limbic cortex can cause mental agitation, panic attack, anxiety. Moreover, $5HTR_{2C}$ agonism induces anxiogenic-like effects in ventral hippocampus and amygdala as $5HTR_{2C}$ overexpression in amygdala. Generally, low 5HT in CNS has been linked to higher risk for anxiety[129].

At the same time, also the endocannabinoid system may be involved in anxiety by suppressing the outflow of glutamate in hippocampus and periacqueductal grey and inhibiting release of NA, 5HT, DA and anxiolytic neuropeptides in corticolimbic areas. On the other hand, the endocannabinoid system interferes with GABAergic transmission to amygdala, hippocampus and prefrontal cortex; all regions involved in the development of anxiety[133]. Pharmacological modulation of cannabinoid receptor 1 (CB1) with its antagonist rimonabant results in anxiogenic behaviours and variants of CB1 receptor gene have been linked in development of anxiety and depression. Also cannabinoid receptor 2 (CB2) appears to be involved in endogenous anxiolytic activity: overexpression of CB2 receptor showed lower anxiety-like behaviours in rats and modifies response to stress[134]. Moreover, endocannabinoid system is able to regulate hypothalamus-pituitary axis (HPA), whose chronical activation leads to anxiety and depression in humans[134]. However, endocannabinoid role in anxiety is still not so clear: treatment with analogues of anandamide in the prefrontal cortex lead to anxiolytic like response due to transient vanilloid receptor 1 (TRPV1) stimulation while fatty acid amide hydrolase (FAAH) carbamate irreversible inhibitors and mono acil glycerol lipase (MAGL) inhibitors have been proved to exert anxiolytic

effect through endogenous acylethanolamides and 2-arachidonoylglicerol (2-AG) concentration rise[134].

4.2 DEPRESSION:

Differently from anxiety, lifetime risk of depression is 15-18%; this means that one in five people ever experiences depression during life, with an average age of onset of 25 years. Depression appears to be twice as common in women than in men. In the majority of cases, depression is episodic, even if long-term depression cases (5-6 years long) have been described in literature[135]. When talking about depression, we refer to a clinical condition characterized by emotional, neurovegetative and neurocognitive symptoms: patients experience anhedonia, depressive mood, feeling of worthlessness and guilt, suicidal ideation, fatigue, alterations of sleep and appetite, indecision and difficulty to concentrate, agitation. To be diagnosed with depression it is necessary that five or more of these symptoms last for more than two weeks. Several conditions may cause depression: environmental factors, stress, pregnancy and parturition, drug administration, anxiety[135].

The physiopathology of depression has been the focus of several studies during time, and different hypothesis have been presented. In particular, increased amount of plasma cortisol, along with changes in the HPA axis have been detected in depressed patients. Moreover, alterations of the neuroplasticity in these areas and reduced amount of brain derived neurotrophic factor (BDNF) have been linked with depression. Also peripheral concentration of cytokines may influence the development of depression: these molecules can reach the brain through its afferent pathways and cross the blood brain barrier causing microglial activation and neuroinflammation as found in brains of patients with depression by post-mortem analysis. Other observations confirming this hypothesis come from patients with autoimmune diseases and severe infections, who are treated with cytokines that are able to trigger depression. Conversely, even if some studies have shown that depression is moderately hereditable, no genetic or epigenetic alterations have been linked to depression yet[135].

However, most accepted hypothesis on depression physiopathology is the monoaminergic one. Talking about DA, researchers discovered that poor functioning of DA neurons may cause depressive symptoms such as hopelessness and loss of interest and that patients with depression show lower levels of DA metabolites in the cerebrospinal fluid[129]. At the same time, also NA can be involved in depression physiopathology: depression has been linked with a decrease of NA in the central nervous system and increased expression and activity of α_2 auto receptor; causing lower noradrenergic neurotransmission. NA decrease in patients has been associated with less pleasure, interest, happiness, alertness, energy[129]. Also, disturbances in 5HT synthesis, release, transport and reuptake may cause depression: lack of 5HT in the brain results in depressive symptoms and may enhance negative emotions. Serum 5HT concentrations in depressive patients are lower compared to controls, and post mortem analysis showed lower tissue concentration in the brain of both 5HT and 5HIAA. Serotonergic alterations in depression can derive from reduced synthesis or abnormal function of serotonergic receptors: depressed patients present a general reduction of $5HTR_{1A}$ and higher distribution of $5HTR_{1D}$ in globus pallidus; while suicide patients present $5HTR_{2C}$ dysfunction in the PFC[129]. Seen all these evidences, there is no surprise that the most used treatments for depression act on monoamines (monoaminoxidase inhibitors, selective inhibitors of 5HT reuptake)[135].

Furthermore, also the endocannabinoid system has a role in depression: hypofunctional endocannabinoid signalling has been associated with depressive illness[136]. In particular, depressed people have high incidence of polymorphisms in CB2 receptor and CB2 overexpression has been linked with decreased depressive-like behaviours. Moreover, endocannabinoids play a pivotal role in neurogenesis, whose alteration has been recognized as one of the main causes of depression. All the evidences above suggest the endocannabinoid system as a possible target of new antidepressant drugs[134].

4.3 RELATIONSHIP BETWEEN OBESITY, ANXIETY AND DEPRESSION

Seen the spread diffusion of obesity, anxiety and depression it's not a surprise the fact that the pathologies can coexist in the same person; but a fundamental question

is: can anxiety and depression be considered obesity comorbidities? Is there a functional link between overeating and the development of mental health diseases?
As explained in other paragraphs, overeating is one of the most important characteristics of obesity and it's mainly caused by the excessive availability of high-processed high-palatable foods. However, sometimes the consumption of these foods is due to the incapability of the individual to overcome negative states deriving from the mind: in this case it's possible to talk about emotional eating (EE)[137]. Of course, one of the first interventions against obesity is subjecting patients to calorie-restricted diets, with the purpose of losing weight: this approach tends to be unsuccessful because of EE, and in particular because of the incapability of obese individuals to control eating in response to stress and negative emotions[138]. When on diet, the individual is abstinent from comfort food and the body is unable to understand the difference between starvation and self-deprivation; thus causing a series of negative emotions that drive the person to abandon the diet[139]. Normally, typical stress response does not involve eating, because physiological stress reactions mimic the sensations associated with satiety, but dieters tend to lose their control on their feelings of satiety and hunger, thus driving them to EE[138].

Even if EE is more common in dieting, it can happen independently, as a response to alexithymia (incapability to identify and describe feelings), poor interoceptive awareness, poor emotion regulation strategies. As expected, EE can be experienced in response to stress, childhood abuse, depression: in this case, the HPA axis may respond atypically with an hypoactivation causing enhanced appetite and weight gain. A lowered HPA axis also explains why emotional eaters are more receptive towards the reinforcing value of food and use it as a medication to overcame negative emotions[138].In addition, EE tends to occur with external eating (overeating in response to food cues, such as sight and smell of attractive foods) since people achieve to shift their attention from negative thoughts to the imminent reward of food[140]: studies have demonstrated that overeating happens only in high anxiety – high food salience conditions and that negative circumstances can enhance the rewarding effects of food. Moreover, results from other researches show that the association of depression diagnosis and severity of depression with emotional eating is significant[138].

Based on these premises, it is not surprising that obesity has an impact on mental health and that there could be a reciprocal, bidirectional link between this pathology and anxiety. Increased risk in anxiety for obese people concerns not only adults, but also children and adolescents, who present higher risk of developing anxiety, depression and mood disorders when obese; maybe due to obesity-associated health problems (diabetes, metabolic syndrome)[141]. Social factors, genetic, epigenetic factors along with CNS alterations can try to explain the high incidence of anxiety in obese patients. As a matter of fact, exposition to maternal high-fat diet and follow up with the same one, can cause the development of an anxiety-like phenotype in macaques and rats due to long term alterations to HPA axis, increased corticosterone-mediated response, alterations of the reward system and of the serotonergic one[141]. In rats, also post-natal overfeeding has been linked with anxiety-like behaviour, and the same has been showed in childhood and adolescence. On the other hand, adult rats exposed to high-fat diet for long periods of time displayed increased anxiety-like behaviours in open field and elevated plus maze paradigms only when exposed also to a stressor agent (for example cat smell or abstinence from the palatable diet), indicating that hypercaloric diet consumption per se may confer a vulnerability to anxiety following exposure to acute stressors, potentially through an increased baseline in HPA axis / glucocorticoid response[141]. Other mechanisms that could be involved in obesity-driven anxiety are hippocampal atrophy, decreased blood vessel density, reduced neurogenesis and synaptic plasticity along with high level of circulating cytokines and impairments in prefrontal cortex and amygdala functional connectivity[141]. On the other hand, clinical studies have failed in correlating BMI and anxiety (both for women and men) and contradictory results have been obtained trying to correlate obesity and anxiety in both sexes; but not a large number of studies was found with the aim to correlate anxiety and obesity in humans[142].

Differently from anxiety, epidemiological evidence supports an association between obesity and depression[143]; literature review studies shows that obese people have 55% of possibility to become depressed[144]. Depression tends to be associated with a reduction of food intake, however a sub-group of patients presents an association between depression and body weight gain; whose mediator was found to be EE[138].

Moreover, depression and obesity are commonly co-occurring conditions, both caused by dysregulation of stress system and characterized by chronic inflammation: adiposity in obesity is a source of cytokines that can promote neuroinflammation as well, increase cortisol, leptin, insulin levels dysregulating the HPA axis and monoaminergic neurotransmission, thus worsening depression[143,145]. In addition, numerous studies have confirmed that overweight/obesity (especially when accompanied by adverse metabolic profile[143]) is a risk factor for developing depression and interestingly demonstrated that treatment of obesity (i.e. by bariatric surgery) and consequent weight loss can improve depressive symptoms[145]. At the same time, other clinical trials using bupropion for the treatment of depression show in obese people significant induction of weight loss, as compared to placebo, and improvements also in the metabolic profile (insulin resistance, serum total cholesterol); although different antidepressants can give different results[145]. Several mechanisms are shared between depression and obesity: first of all, genes near BMI-associated loci are highly expressed in brain regions involved in energy homeostasis and mood regulation (hippocampus, hypothalamus and limbic regions)[143]. Moreover, genetic loci reliably associated with depression phenotypes overlapped or were closer to genes associated with BMI and early-onset obesity (gene NEGR1 for example). Other genome-wide association studies confirmed that the polygenic architecture of depression is partially superimposable to obesity-related traits[143]. In addition, as mentioned for anxiety, both depression and obesity are associated with HPA axis and reward system impairments. Furthermore, leptin resistance (common in obese people) may constitute a phenotype risk for depression and insulin dysregulation (also characteristic in people with the pathology) may play a role in neuropsychiatric conditions[143]. Also microbiota alterations noticed in obesity have an emerging role in the relationship between the pathology and depression, since relate with impaired gut permeability, inflammation and depression-related brain processes[146].

5. NEUROINFLAMMATION AND OBESITY

As stated before, obesity is characterized by a chronic state of inflammation: excessive fat becomes an endocrine organ capable of producing hormones that can dysregulate several pathways and lead to comorbidities, such as endocrine disfunctions, incremented cancer risks, atherosclerosis, depression. For example, the inflammatory state produced by obesity induces peripheral binding of leptin with reactive protein C, decreasing its physiological efficacy and inducing alterations to the control of food intake and satiety[147].

Adipose tissue produces chemokines (such as MCP-1 and CCL-2) which recall monocytes causing macrophage activation and cytokine hyperproduction[148]. Several cytokines can cross bidirectionally the BBB: in particular interleukins, tumoral necrosis factor alpha (TNF-α), leukaemia inhibitory factor (LIF), adipokines that act as mediators of inflammation and neurodegenerative processes, induce cellular death, apoptosis and mediate the release of other cytokines. In CNS, cytokines activate neuroendocrine system, vary metabolism and neurotransmitters function, alter neural plasticity and cerebral circuits and contribute to the development of dementia, cerebral ischemia[149].

A lot of animal models of obesity show that even few days of high-fat diet exposure can induce fat acid increment in blood, which can cross the BBB and provoke inflammation in hypothalamic neurons. Such local inflammation in hypothalamus involves ARC, PVN and median eminence (ME) and induces endoplasmic stress in neurons, leading to leptin and insulin resistance development[150]. In adult male rats, an increment of immune cells from periphery to CNS has been observed, in particular in hypothalamus[151].

Several neuronal cells are involved in inflammation and neuroprotection. Between them, microglial cells maintain synaptic plasticity and are fundamental during development and neurogenesis. Microglial cells activation normally has a protective action on CNS, but chronic and uncontrolled activation can be harmful: in obesity microglial cells are active in the hypothalamus and secrete cytokines that recall immune cells from periphery[152]. Macrophage cells in hypothalamus release

fractalkine and activate CX3CR1 receptor sustaining neuroinflammation[153]. In obesity, saturated and unsaturated fatty acids can activate microglial receptor pathway of tool-like receptor 4-NF-kβ, raising cytokines and oxygen reactive species (ROS) production[154].

Also glial cells have a pivotal role in neuroinflammation. Not active glial cells have a small cell body and ramified terminations that monitor all CNS areas; in their active state they are capable of producing inflammatory mediators, phagocytize other cells and present antigens[155].

Astrocytes are another class of cells that mediate neuroinflammation: their activation has been noticed in cerebral trauma, ischemic shock, neuroinflammation; producing IL-6 and TNF-α and regulating BBB and blood vessels permeability[155].

Along with astrocytes, pericytes modulate BBB homeostasis: these contractile cells enclose endothelial cells of capillaries and, when activated by immune system, produce cytokines and ROS, act on tight junctions incrementing BBB permeability[156].

Other cells involved in immune response are tanicytes and polydendrocytes that regulate energy homeostasis and at the same time have a role in BBB permeability modulation.

In neuroinflammation caused by obesity, hypothalamus is the most impacted area so far. However, recent studies suggest increased levels of tool-like receptor 4, cyclooxygenase-2 (COX-2), interleukin and iNOS in rats hippocampus after long-term exposure to high-fat diet[157]. Also, in hippocampus is possible to appreciate a reduction of GLUT1 receptor, linked with memory and cognition[155].

In the same animals, several evidence of cytokine and chemokine production, increased levels of COX-2 and prostanoids and reduced dendritic density were found in cortex, along with microglial and astrocyte activation[158].

Even in brainstem, nucleus solitary tract and cerebellum exposition to high-fat diet provoked increased expression of interleukin, NF-kβ, TNFα.

All this evidence, taken together, suggests that neuroinflammation is a common condition occurring with obesity and that it can be responsible of some of the impairments associated with obesity comorbidities.

6. THE ENDOCANNABINOID SYSTEM

Psychoactive effects of cannabis have been known for centuries by human society, but the discovery of an endocannabinoid system only happened in 1990, when Matsuda et al. firstly cloned CB1 in the brain[159]. A second peripheral receptor (CB2) was later identified by Munro et al[160]; and respectively in 1992 and 1995 also endocannabinoid agonists anandamide (AEA) and 2-AG were discovered[134].

The endocannabinoid system is made up by endocannabinoid receptors, ECs and their enzymes of synthesis and degradation.

Specifically, CB1 receptor was first isolated in the CNS, where it is mainly expressed; as one of the most abundant G protein-coupled receptors. In rodent brain, CB1 is mostly expressed in basal ganglia, SN, globus pallidum, cerebellum, hippocampus, brainstem, where it regulates appetite, learning, memory, mood and reward[161]. Both GABAergic and glutamatergic neurons express CB1 receptors; thus giving to the endocannabinoid system an important role in modulating neurotransmission[134]. Principally, CB1 receptors are found presynaptically, where they inhibit neurotransmitter release. Differently from CB1, CB2 receptor was thought to be expressed only in immune cells. Only in the last years, CB2 expression was studied also in the CNS in particular in microglial cells. It seems possible that CB2 receptor is part of a general neural protective system[134].

Both CB1 and CB2 are coupled to a $G_{i/0}$ protein, so their activation decreases the production of cyclic AMP (cAMP) and inhibits protein kinase A (PKA); stimulates the mitogen-activated protein kinase (MAPK), P38, Rho kinase, ROCK; affecting synaptic plasticity, cell migration, neuronal growth. Both receptors can be phosphorylated by G protein receptor kinases (GRK) and associate with β-arrestin; leading to receptor internalization and ERK pathway activation[162].

Figure 1 shows pathways of CB1 and CB2 activation.

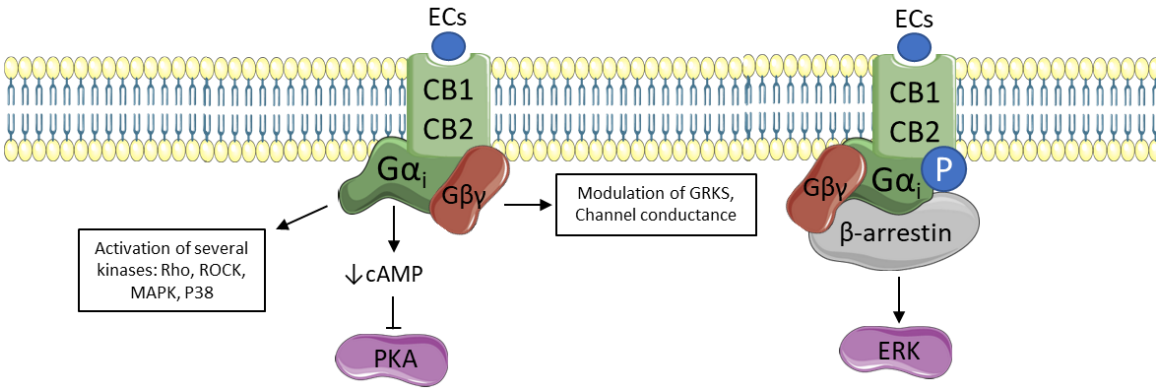

Figure 1: CB1 and CB2 signalling pathways.

Other receptors that are suggested to mediate endocannabinoid action are the orphan G receptor GPR55 and TRPV1; whose binding essays showed affinity both for 2-AG and AEA[163].

CB1 and CB2 receptors are activated by the endocannabinoids 2-AG and AEA. Differently from other neurotransmitters, these molecules are small lipid signals synthetized "on demand" and are not stored in lipid vesicles in neurons; moreover they act mostly pre-synaptically rather than post-synaptically[163].

2-AG synthesis takes place from 2-arachidonoyl-containing diacylglycerols (DAG); thanks to enzymes diacylglycerol lipase (DAGL) α and β. Several studies demonstrated that DAGLα is the most abundant isoform in the brain; DAGLβ expression was noticed in the same tissue but it's more abundant in the periphery. DAG precursors come from hydrolysis of membrane phospholipids thanks to PLC or from phosphatidic acid hydrolysis[163].

Brain 2-AG levels are 170 times higher than those of AEA; activation of the CB1 and CB2 receptors by 2-AG is associated with many physiological processes, including inflammation, food intake, locomotor activity, learning and memory, epileptogenesis, neuroprotection, pain sensation, mood, stress and anxiety, addiction and reward[164].

2-AG degradation occurs thanks to the enzyme MAGL, expressed in synaptic terminals. In certain conditions, 2-AG can be oxidised also by cyclooxygenase 2 (COX2) to produce prostanoids, important mediators of inflammation[165].

Figure 2 shows synthetic and degradation pathways of 2-AG.

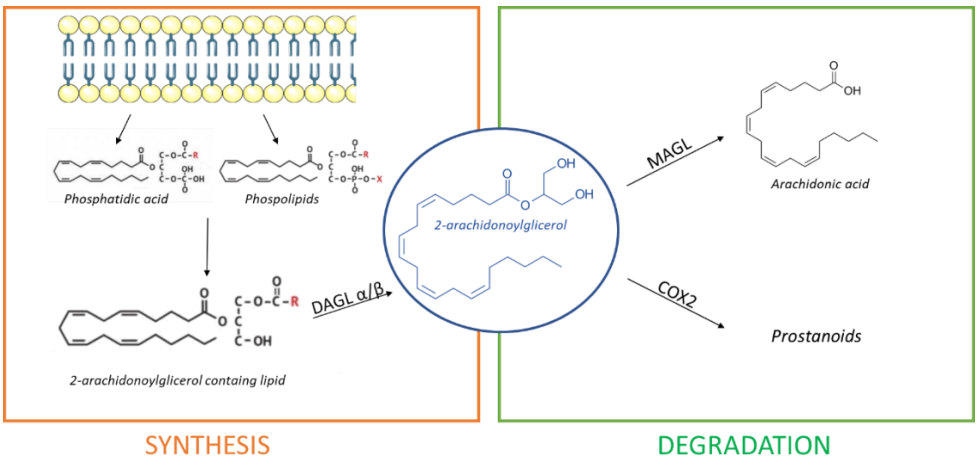

Figure 2: 2-AG synthetic and degradation pathway.

AEA synthesis can occur in three different ways:

- Directly from the hydrolysis of N-arachidonoyl-phosphatidyl ethanolamines (NArPE) through enzymatic action of N-acyl phosphatidylethanolamine phospholipase D or NAPE PLD;
- Through NArPE deacetylation by α/β-hydrolase domain type-4 (ABHD4) and hydrolysis of glycerophosphoethanolamine by glycerophosphodiesterase GDE1;
- via PLC-mediated hydrolysis of NArPE to yield phosphoanandamide, which is dephosphorylated to AEA by a phosphatase.

The most common pathway is the first described[163]. Several studies indicate that AEA exerts an overall modulatory effect on the reward circuitry[166], neuroinflammation, microglial activation, central control of blood pressure, pain modulation[167].

AEA degradation is catalysed by the enzyme FAAH[165]. Inhibition of FAAH is able to increase the endogenous tone of acylethanolamides[133]; thus involving important therapeutical and experimental implications. Also, AEA can be metabolized by COX-2 giving prostanoids; or alternatively it can be hydrolysed by N-acylethanolamine-hydrolysing acid amidase (NAAA)[165]. Differently from 2-AG, AEA is only a partial agonist of CB1 receptors[161].

Figure 3 shows AEA synthetic and degradation pathways.

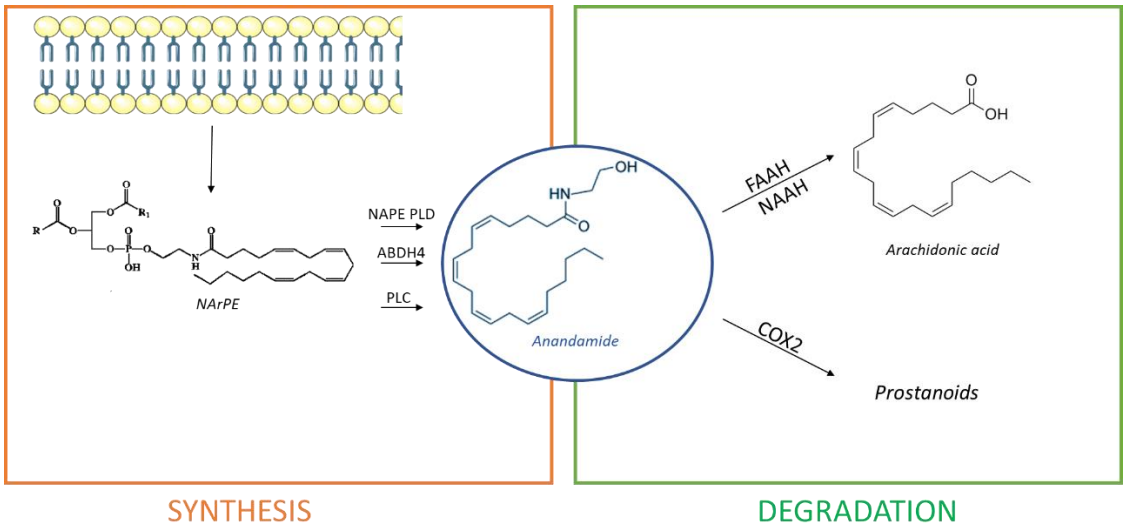

Figure 3: AEA synthetic and degradation pathway.

Other important lipid mediators, which do not show particular affinity towards CB1 and CB2 receptor but that share AEA biosynthetic and degradation pathways are palmitoilethanolamide (PEA) and oleoylethanolamide (OEA).

PEA is produced both in the periphery and in the CNS and has anti-inflammatory and pain-modulating effect[163]; it has also analgesic, antiepileptic and neuroprotective actions[161]. PEA actions are due to its binding to GPR55 and PPARα receptor[134].

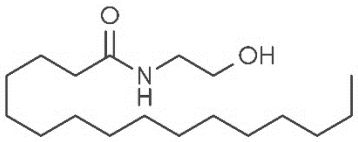

Figure 4: PEA structure.

Like PEA, OEA is produced in intestine and in the CNS; it is known for its hypophagic[168] and neuroprotective effect[169]; due to its binding with PPARα and TRPV1 receptors[168].

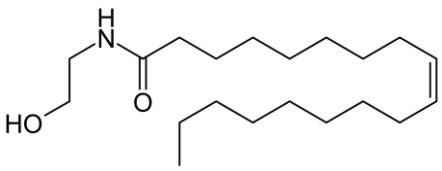

Figure 5: OEA structure.

All these lipid mediators regulate food intake and are sensors of whole-body energy status and can act on hypothalamus modifying leptin signalling: higher endocannabinoids levels have been associated with defective leptin; whereas OEA acts as a satiety factor engaging brain histamine[123].

In the brain, the endocannabinoid system plays numerous functions: it regulates learning, memory and cognition; motor activity; mood tone; appetite and food intake; reward and addiction; neuroprotection; neural development and sleep[170].

Pathological alterations of the endocannabinoid system have been noticed in nearly all chronic disorders and in particular in addiction, neurodegenerative diseases, mood disorders[163] and obesity. Referring to obesity, it is important to know that the endocannabinoid system has an important role in regulating food intake: orexigenic effect of endocannabinoids depend on the stimulation of CB1 receptor of glutamatergic neurons in the cortex; whereas anorexigenic effects can be attributed to CB1 activation in GABAergic neurons of DLS[171]. Fasting increases endocannabinoid concentrations within the ACC, activating the release of DA and driving liking and motivation to consume palatable foods[172]. Cannabinoid receptors in this area decrease the release of glutamate on GABAergic neurons projecting to ventral tegmental area (VTA): activation of these receptors turns in VTA dopaminergic neurons disinhibition[173]. CB1 receptors are also expressed on hypothalamic neurons controlling food intake, such as POMC expressing neurons: in these cells CB1 receptor stimulation causes release of the orexigenic peptide β-endorphin and inhibits the release of anorexigenic peptide α-MSH[174]. On the other hand, leptin is able to reduce cannabinoid levels in the brain and interfere with endocannabinoid system signalling. Additionally, the endocannabinoid system has a strong relevance in the gut-brain axis: cannabinoid receptors are expressed on peripheral terminals of sensory neurons; in parasympathetic and sympathetic terminals; on vagal afferences and efferences regulating gastric motility[175].

Obesity has been linked to an overactive endocannabinoid system[176]. Plasma endocannabinoids correlate positively with markers of obesity and metabolic disorder: in particular variations in blood concentrations of AEA were noticed when comparing fat and lean individuals, suggesting that its dysregulation in obese patients

can contribute to the increased food intake[177]. In addition, intake of high quantities of fat with the diet impairs endocannabinoid and other lipid signal production; affecting in particular inflammation and synaptic plasticity[176]. CB1, CB2 and FAAH genetic alterations have been associated respectively with metabolic syndrome, overweight and obesity[175,178]. Carriers of these polymorphisms have higher levels of circulating endocannabinoids and show greater reward-related striatal activity[175]. Likely, this genetic variation may influence the propensity to develop obesity depending on the diet: rodents with these genetical variations display increased body weight and metabolic variations only when fed with high fat diet and not with chow[179].

Seen all the physiological processes in which the endocannabinoid system is involved, the same has been targeted to produce effective drugs against various pathologies: in particular, CB1 agonists have been used as antiepileptic drugs, neuroprotective in Huntington's disease, Alzheimer's, Multiple sclerosis; CB1 antagonist Rimonabant was used as anti-obesity treatment (then dismissed due to adverse psychological reactions); FAAH and MAGL inhibitors have been investigated as antiepileptic drugs and neuroprotective in neurodegenerative diseases[163].

For the aim of this thesis, it is strongly important to focus on FAAH inhibition: several animal studies used FAAH inhibitors successfully in depression, anxiety, sleep disorders, neuropathic pain. However, up to date no FAAH inhibitor has been approved for use in humans and several clinical trials involving these drugs have been suspended due to important adverse reactions[180].

PF-3845, the drug used in this study, is an irreversible FAAH inhibitor; characterized by its selectivity, elevated oral bioavailability and lower toxicity[181]. The drug is a biaril-ether-piperidine derived and acts creating a covalent and irreversible bind to S241 of FAAH enzyme. Its administration results in increased acylethanolamide tone for 24 hours[133]; it is able to produce antialgesic, anti-inflammatory[182], antidepressant and anxiolytic effects[183].

7. CENTRAL MONOAMINERGIC NEUROTRANSMITTERS: DOPAMINE

Discovered by Carlsson et al in 1957, dopamine (DA) is a chemical neurotransmitter of the brain[184]. As other monoaminergic transmitters, it is a small charged molecule, normally unable to cross the BBB[185] and synthetized from amino acids by regulated enzymatic reactions. DA has essential roles in regulating movement[186], spatial memory function[187], motivation[188], arousal[189], reward [190], lactation[191], sexual behaviour[192], and nausea[193], etc.

DA is synthetized from tyrosine (an amino acid introduced with diet) in a two-step reaction that takes place in the cytosol of dopaminergic neurons. The enzyme tyrosine hydroxylase (TH, the rate-limiting enzyme of the reaction) converts tyrosine in levodopa (L-DOPA) using oxygen and iron as co-factors[194]. Then, L-DOPA is converted in DA by the enzyme DOPA decarboxylase (DDC). Other synthetic pathways can occur: for example DA could be indirectly synthetized from phenylalanine[195] or, in SN, from p-tyramine through cytochrome P450 2D6 (CYP)[196]. After being synthesized, DA is stored in vesicles via the vesicular monoamine transporter (VMAT) until firing. Moreover, in adrenergic and noradrenergic neurons, DA could further be processed in norepinephrine and epinephrine by the enzyme DA-beta-hydroxylase (DBH)[197].

Usually, DA is metabolized by enzyme monoamino-oxydase B (MAO-B) into 3,4-Diidroxyphenylacetaldehyde (DOPAL), further processed by enzyme aldehyde dehydrogenase (ALDH) in 3,4-siidroxyphenilacetic acid (DOPAC). DOPAC can be further metabolized in homovanillic acid (HVA) by enzyme catechol-O-methyltransferase (COMT)[194]. When not stored in vesicles or metabolized by MAO and COMT, DA undergoes spontaneous oxidation, generating DA quinones, which are more toxic species[194]. DA quinones polymerize and form neuromelanin, a dark pigment that gives its characteristic colour to SN[198].

Figure 6 shows DA pathways of synthesis and degradation.

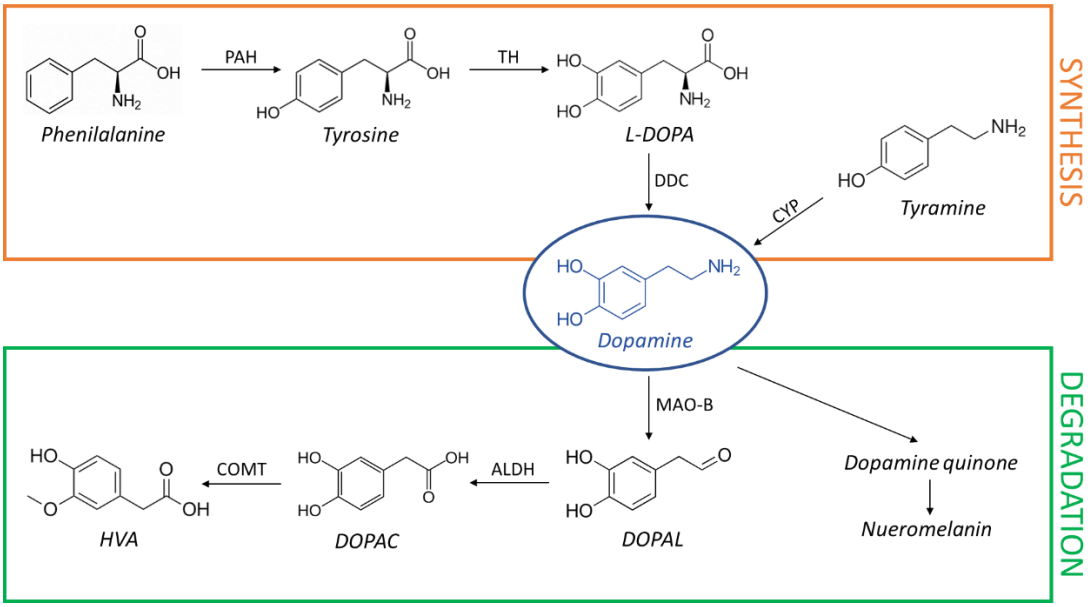

Figure 6: Dopaminergic pathways of synthesis and degradation.

DA release is mediated principally by two different mechanisms: phasic and tonic release. Phasic release occurs in response to action potential in dopamine-containing cells; whereas tonic release happens independently from presynaptic action potentials and it is regulated by the activity of other neurons[199]. After being released in the synaptic cleft, DA is internalized by selective reuptake mediated by DA transporter (DAT) and metabolized.

Five different types of DA receptors have been sequenced until now: D1, D2, D3, D4, D5. All types of DA receptors are metabotropic (G-protein coupled) and lead to the formation of second messengers, which can activate or inactivate specific signalling pathways[194].

Principally, DA receptors are widely expressed in the central nervous system, but can also be found in retina, blood vessels, heart, kidney, adrenal glands, gut; where they control catecholamine release and the renin-angiotensin system[200]. In the brain, D1 and D2 type receptors are the most abundantly expressed, and rarely co-expressed on the same cell[200].

DA receptors are subdivided into two different families: D1-like (receptor D1 and D5) and D2-like (receptor D2, D3, D4). D1-like receptors are expressed mainly in the striatum, ACC, SN pars reticulata, AMY and frontal cortex; whereas D2-like receptors

are expressed in striatum, globus pallidum, nucleus accumbens core, VTA, hypothalamus, AMY, cortical areas, hippocampus, pituitary gland[201]. Moreover, D1-like receptors are coupled to $G\alpha_s$; while D2-like receptors are coupled to $G\alpha_i$. Another difference between the two families of receptors is that DA has from 10 to 100-fold greater affinity for D2-like receptors, as compared to D1-like. Additionally, several studies demonstrated that DA receptor can dimerize with other receptors (for example there is evidence of D2-D4 dimers, or D1-NMDA ones) which have complex and different signalling properties[194].

When DA binds to D1-like receptors, since they are coupled to $G\alpha_s$ proteins, it activates protein adenylate cyclase, which produces higher levels of cAMP that stimulate the activity of protein kinase A (PKA). Differently, when a D2-like receptor is activated, since it is coupled to $G\alpha_i$ protein, there is the inhibition of adenylate cyclase, less production of cAMP and inhibition of the activity of PKA. PKA has lots of targets, including cAMP response element-binding protein (CREB), glutamate receptors, GABA receptors, ion channels[194]. Another target of PKA is the dopamine and cAMP regulated phosphoprotein 32-kDa (DARPP-32), which is able to amplify PKA signalling and integrate or modulate signalling pathways of numerous neurotransmitters[202]. DARPP-32 inhibits protein phosphatase 1 (PP1), which is responsible of histone dephosphorylation, thus promoting a phosphorylated state in the cell and enhancing PKA-mediated signalling. PP1 inhibition inactivates striatal enriched tyrosine phosphatase (STEP) dephosphorylation, which does not dephosphorylate extracellular-signal regulated kinases (ERK) that has a role in cell death and development. ERK could also be activated by D2-like receptors with a mechanism that is still unclear[194]. At the same time, D2-like receptor activation increases the concentration of Ca^{2+}, and promote calmodulin-dependent protein phosphatase 2B (PP2B), which can dephosphorylate DARPP-32[203]. DARPP-32 may interact with different proteins, hormones, and neurotransmitters[202].

$G_{\beta\gamma}$ subunit of both receptors D1 and D2-like has been shown to participate in the modulation of GABA receptors, brain derived neurotrophic factor (BDNF) activation and several ion channel modulation such as: G protein inwardly rectifying potassium channels (GIRKs), L and N type Ca^{2+} channels, Na^+/K^+ ATPase[194].

Other dopaminergic receptors may also couple with $G\alpha_q$ protein (D5, D1-D2 dimers), which results in phospholipase C activation (PLC) and production of diacylglycerol (DAG) and inositol triphosphate (IP3); which result respectively in incrementing Ca^{2+} concentration and activation of protein kinase C (PKC). These activations result in PP2B and calcium calmodulin dependent protein kinase II (CaMKII) pathways regulation[194].

Other G protein coupled receptors can modulate dopamine receptors: in particular G protein coupled receptor kinases GRK2 and GRK3 can phosphorylate D1, D2 and D3 receptors. In particular, D2 receptor phosphorylation results in its binding to β-arrestin and internalization. The complex D2 receptor-βarrestin is capable to inactivate PI3K-AKT signalling (linked to cell survival, proliferation, glucose metabolism, gene transcription) and activate GSK3 pathway (related to circadian response and reward behaviour)[194].

Figure 7 shows most important pathways for DA signalling.

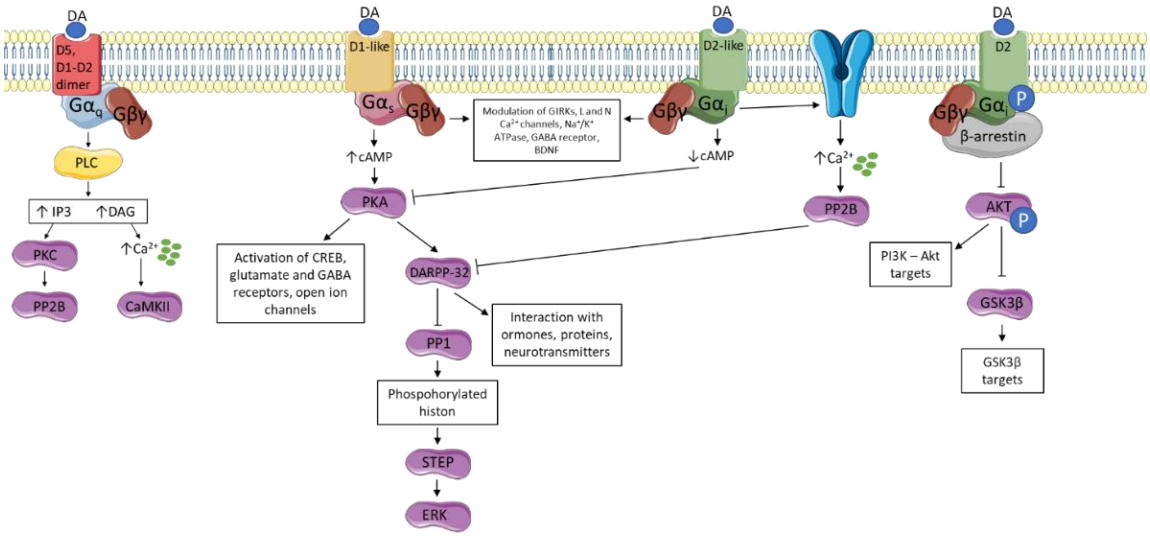

Figure 7: Dopaminergic signalling pathways.

As mentioned before, DA has fundamental roles in neuromodulation, motivation, reward, cognitive function, feeding and influences both the immune, cardiovascular, gastrointestinal and renal systems; all these actions are exerted thanks to central pathways. In the brain, DA is mainly synthetized in VTA and SN; which are the two

main brain areas where dopaminergic cell nuclei reside. From VTA and SN, dopaminergic neurons release projections to other brain areas. In particular, we can talk about a mesocortical pathway (from VTA to PFC) and a mesolimbic pathway (from VTA to ACC), which combine in the mesocorticolimbic system that has a fundamental role in reward and motivation, appetite-motivated behaviours, determination of personality traits. Mesocorticolimbic system is fundamental also in regulating food intake: food exposure leads to DA release in these regions and high-palatable food consumption causes DA neuron firing from VTA to ACC. Disfunction in the mesocorticolimbic system have been related to drug addiction, reward impairments, mood disorders[204].

From VTA other projections reach amygdala, hippocampus, cingulate gyrus and olfactory bulbs[194].

On the other hand, neurons departing from SN project to the DLS, forming the nigrostriatal pathway; principally involved in controlling movement, motivated behaviours, central pain modulation[194].

Another pathway involving DA is the tuberoinfundibular: it is composed by projections of DA neurons from ARC and PVN to pituitary gland; this pathway is involved in the control of the secretion of prolactin[194].

The less investigated dopaminergic pathway is a diencephalic cluster in the dorsal posterior hypothalamus (known as A11 region), which projects to neocortex, serotonergic dorsal raphe (DR), dorsolateral funiculus; controlling ascending sensory information, cardiovascular and sympathetic activity[194].

Figure 8 shows dopaminergic pathways in CNS.

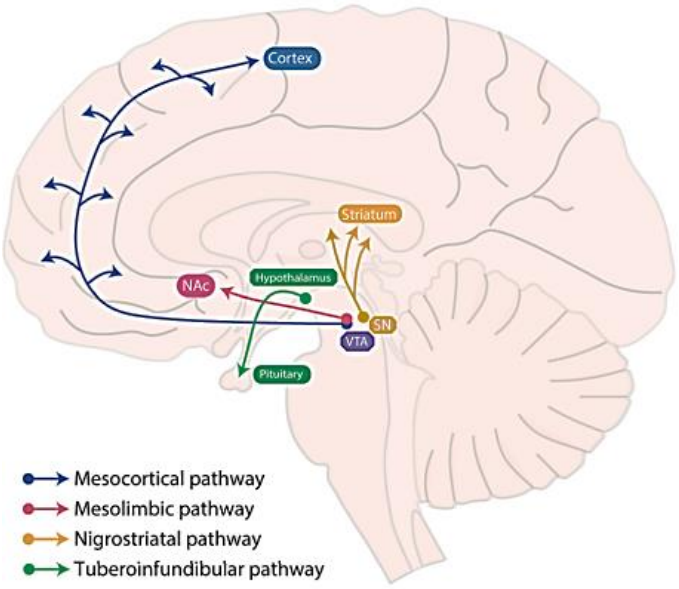

Figure 8: Dopaminergic pathways in CNS. Image was extracted from Klein et al[194].

Numerous diseases involve dopaminergic system disruption: between them, we can find Parkinson's disease, Huntington's disease, schizophrenia, attention deficit, hyperactivity disorder and addiction[194]; at the same time numerous pathological conditions can disrupt the dopaminergic system and, between them, we can also find obesity.

Several studies have been investigating the role of the dopaminergic system in obesity, and whether dopaminergic impairments could cause obesity or if obesity could cause modifications to the neurotransmitter's pathways: several obesity-related psychosocial (emotional eating, depression) and metabolic (insulin sensitivity) factors have been linked to disturbances in the dopaminergic system[205]. On one hand, in the last years a new hypothesis on obesity pathogenesis has been investigated: the reward deficiency hypothesis states that excessive food intake is necessary to compensate a diminished reward effect of food consumption due to decreased dopaminergic activity[117]. DA has a crucial role in feeding, since transgenic mice that do not synthetise DA die of starvation because of their lack of motivation to eat[206]: in humans, palatable food ingestion causes DA release in DLS in proportion to the self-reported level of pleasure deriving from eating[207]. In mild obesity, decreased tonic/increased phasic DA release was noticed; differently from increased tonic/ decreased phasic DA release in the

progression of obesity pathology[208]. On the other hand, patients with metabolic obesity due to leptin deficiency show major activation of DA mesolimbic targets to visual food stimuli, associated with food wanting, even when the subject had just been fed[204]. Moreover, alterations of normal response to orexigenic and anorexigenic peptides in dopaminergic neurons were detected in these subjects[209].

Preclinical and clinical studies in obesity have provided evidence of reduced DA signalling in the striatum: importantly, a decrease of D2, that in this region has been linked to compulsive food intake; decreased metabolic activity in ACC and orbitofrontal cortex that facilitate hyperphagia, reduced sensibility to rewards compensated with overeating. Moreover, a negative correlation between D2 and BMI was reported in obese and overweight people[204]. In the same area, blunted striatal DA concentrations have been described in numerous studies and low striatal DAT levels were detected[209]. In addition to lower D2 availability, weight gain has been associated with genetic differences in D2, in Taq1 allele of ANKK1 gene[209]. Moreover, D4-L allele modifications have been associated with higher BMI[210]. Polymorphisms in DA receptors D2, D3, D4; in DA transporter (DAT1), in enzymes of DA degradation (COMT, MAO) have been related with obesity[211]. At the same time, evidence suggests that increased consumption of high-fat foods causes decreased DA activity in VTA[212]. Moreover, diet-induced obesity has been linked to decreased TH expression in DA relevant brain regions[209]. All this evidence of decreased activation of DA in response to food intake might be the reason of overconsumption, which takes place in order to compensate weak DA signals[213].

8. CENTRAL MONOAMINERGIC NEUROTRANSMITTERS: NORADRENALINE

Another important central neurotransmitter is noradrenalin or norepinephrine (NA), discovered in the early ‘40s by Ulf von Euler[214]. Central NA is produced in locus coeruleus (LC) and it’s involved in learning, anxiety, pleasure, arousal, attention, stress[215,216].

NA shares the same biosynthetic and degradation pathways of DA; since it's synthetised from the amino acid tyrosine. Tyrosine goes through hydroxylation and decarboxylation, giving DA; which is further oxidated by enzyme DBH to produce NA. Once produced, NA is stored in vesicles thanks to transporters VMAT-1 and VMAT-2. Due to neuron stimulation, NA is released in the synaptic cleft; and then reuptake by norepinephrine transporter (NET) into the presynaptic neuron or metabolized by enzyme COMT and MAO, likewise DA. In the preferential way of degradation, NA is converted in NA aldehyde by MAO; then reduced by ALDH in 3,4-dihydroxyphenilglycol and processed by COMT in 3-Methoxy-4-hydroxyphenyl glycol (MOPEG)[216].

Figure 9 shows synthesis and degradation pattern of NA.

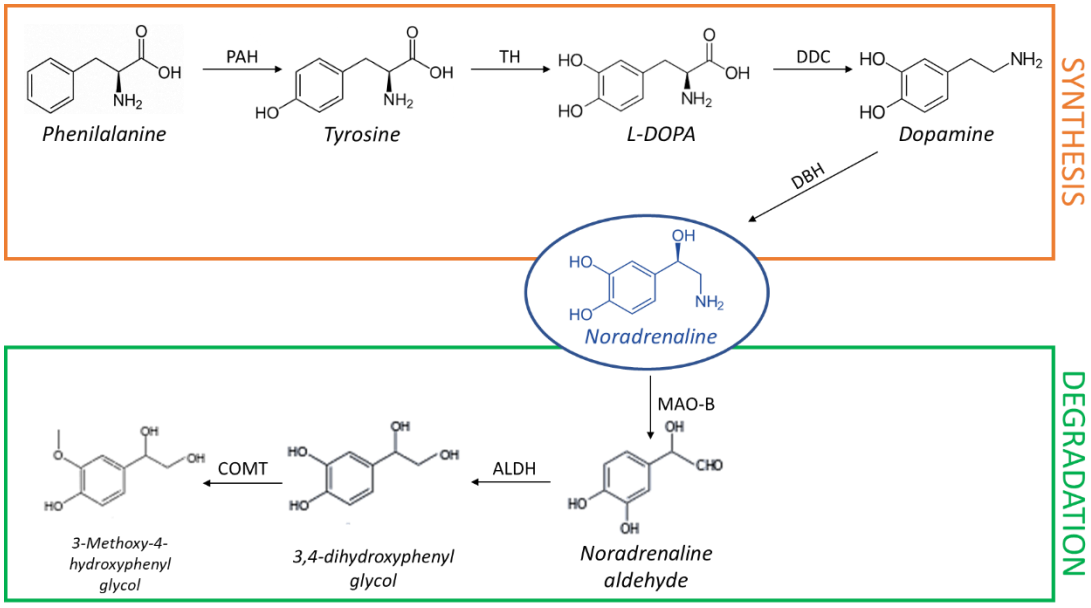

Figure 9: Noradrenergic pathways of synthesis and degradation.

Like DA, NA release can happen in two distinct modes: tonic and phasic. Tonic activity is typical of wakefulness, decreases during slow-wave sleeping and ceases during REM sleep. On the other hand, phasic release happens during focused attention and accurate task performance[216].

Central actions of NA are promoted by its binding to noradrenergic receptors. All noradrenergic receptors are coupled to G protein, and can be subdivided into three

different families: α_1, α_2, β. In general, excitatory effects are mediated by α_1 and β receptors; whereas inhibitory ones are mediated by α_2. α_1 receptors are linked to G_q sub-unity; their activation results in PLC activation, IP3 and DAG production; increased intracellular Ca^{2+} concentration, reduction of potassium conductance and increased cell excitability. Principally, these are postsynaptic receptors in hippocampus, thalamus, striatum, raphe, VTA. Differently, α_2 receptors are coupled to $G_{i/0}$ protein; their activation inhibits adenylate cyclase and results in increased potassium conductance, decreased intracellular Ca^{2+} concentration, reduced neurotransmitter release. These receptors are found as somatodendritic autoreceptors in LC, or presynaptically in noradrenergic and non-noradrenergic terminals.

β receptors are coupled to G_s; their activation stimulates adenylate cyclase, cAMP, PKA cascade and results in promoting repetitive discharge, facilitating long term potential. Principally, they are found in cerebellum, blood vessels and astrocytes.

Figure 10 shows noradrenergic pathways linked to receptor activation.

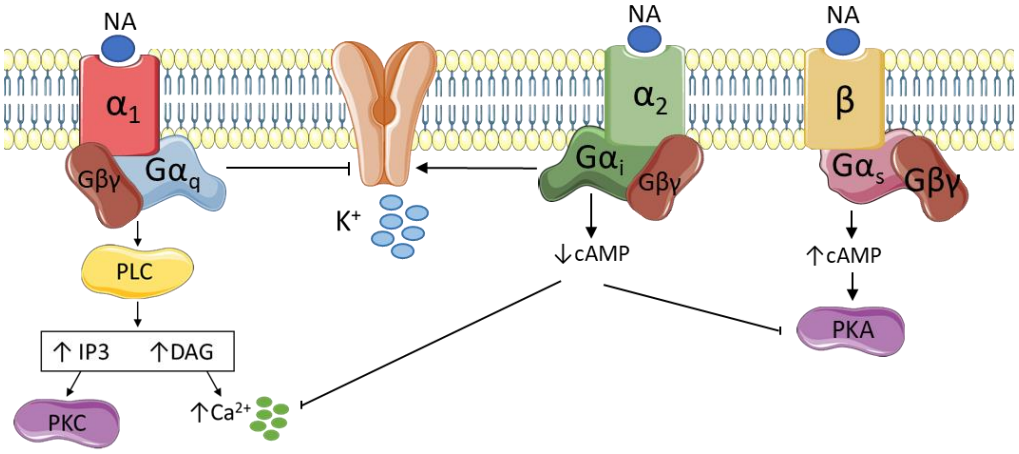

Figure 10: Noradrenergic signalling pathways.

In the brain, NA is produced only in LC. Several neuropeptides are expressed in LC neurons, and so regulate NA release: between them the most important is galanin[217]; whose release modulates wake/sleep states, nociception, feeding and parental

behaviour[218]. LC neurons also express NPY; and some of them may co-express both galanin and NPY[217]. However, NA neurons contain several adrenoreceptor subtypes, mostly α_1 and α_2[219]. Nicotinic acetylcholine receptors are also highly expressed within the LC[214]. Moreover, LC receives afferent innervation from bed nucleus of stria terminalis, cerebellum, central amygdala, cortex, hippocampus, periaqueductal grey. At the same time, several are the projections that NA neurons have throughout the brain, departing from LC: nearly all brain regions present DBH positive axons; except for DA areas. Regions rich in noradrenergic projections are: forebrain, cerebellum, brainstem, spinal cord[214].

NA neurons disruption in LC has been linked to several pathologies: Alzheimer's disease, Parkinson disease, multiple sclerosis, psychiatric disorders, anxiety, depression have been correlated with NA neuron loss, degeneration or alteration[214,216].

More interesting for the aim of this thesis, central NA is involved also in the control of food intake. In particular, noradrenergic fibres form the brainstem project to hypothalamus[220]. Activation of α_1 and β receptors decreases food intake; whereas activation of α_2 receptors increases food intake. In particular, in PVN cells α_1 receptor activation induces excitatory effect that inhibits food intake; whereas α_2 receptor activation has been linked with the disinhibition of descending satiety cells, thus stimulating food intake[221]. A proof sustaining this hypothesis is that the administration of amphetamine (a DA-NA-reuptake inhibitor and releasing agent) has weight reducing effects mediated by increased NA signalling in the hypothalamus[222]. Additionally, is important to say that peripheral NA, as principal neurotransmitter of the sympathetic nervous system, has an important role in controlling energy homeostasis and fat storage for its expression in both white and brown adipose tissue[223].

Up to date, there is not any literature linking obesity and alterations to the central noradrenergic system.

9. CENTRAL MONOAMINERGIC NEUROTRANSMITTERS: SEROTONIN

Serotonin (5HT) was discovered by Italian scientist Vittorio Erspamer in 1937 in enterochromaffin cells of gastrointestinal tract[224]. Only in 1957 Brodie and Shore proposed that serotonin could be a neurotransmitter[225], and evidence demonstrated that 5HT receptors were abundant in the CNS[226]. Since then, several roles have been attributed to central 5HT in the regulation of mood, behaviour, sleep, body temperature, sleep cycles, appetite and also in several pathologic conditions[226].

5HT is a monoamine, such as DA and NA, and it's synthetised from the essential amino acid tryptophan. First, tryptophan is hydroxylated by enzyme tryptophan hydroxylase (TPH) in L-5-hydroxytriptophan; then it's decarboxylated by enzyme L-aromatic amino acid decarboxylase (AADC) in 5HT[227]. Enzyme TPH is specific for tryptophan and is considered the rate-limiting enzyme of the reaction. Mainly, in the brain, 5HT synthesis happens in DR. Here, serotonin is stored in vesicles until neuron firing. Consequently with 5HT release in the synaptic cleft, 5HT can be restored in the presynaptic neuron thanks to selective serotonin transporter (SERT) or metabolized by MAO[228] in 5-hydroxyindolacetic acid (5-HIAA). Alternative pathways of 5HT degradation happen in pineal glands, where it is converted into melatonin[226].
Figure 11 shows serotonin synthesis and degradation pathways.

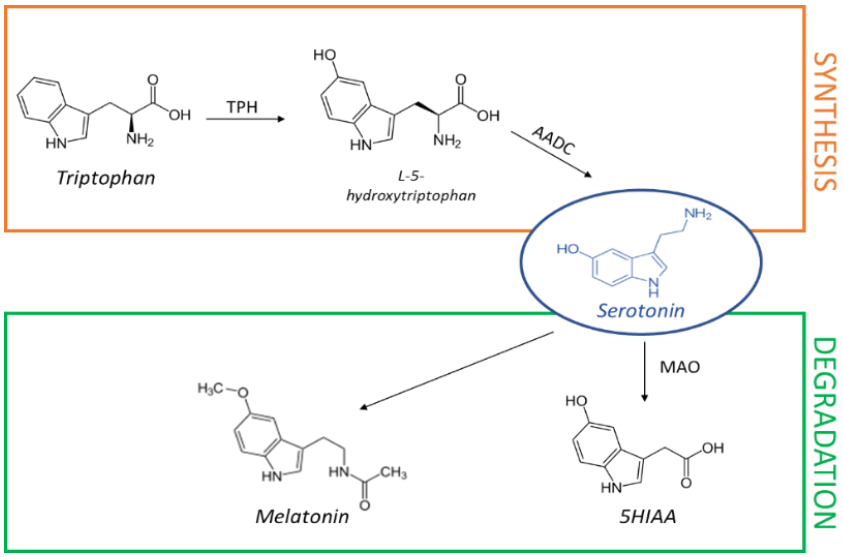

Figure 11: Serotonergic pathways of synthesis and degradation.

5HT explicates its action in the CNS thanks to the binding with its receptors: up to date, seven different types of 5HT receptors ($5HTR_{1-7}$) have been cloned. Exceptionally from receptor $5HTR_3$, all serotonin receptors are G-coupled. In particular, receptors $5HTR_1$ and $5HTR_5$ are coupled to $G_{\alpha i}$ protein; receptor $5HTR_2$ is coupled to G_q protein; receptors $5HTR_{4,6,7}$ are coupled to $G_{\alpha s}$ protein; whereas $5HTR_3$ receptor is a potassium channel[229].

Talking about $5HTR_1$, different subtypes have been cloned until now: $5HTR_{1A}$; $5HTR_{1B}$; $5HTR_{1D}$; $5HTR_{1E}$[230]. Principally, receptor $5HTR_1$ is expressed in DR as a presynaptic receptor, where it inhibits further release of 5HT, or as a post synaptic receptor in PFC and other cortical areas, modulating DA release[231]. Several antidepressant or antimigraine drugs act on these receptor type[229].
$5HTR_2$ is present in three different subtypes (A, B, C) and its activation leads to intracellular calcium increase[229]. $5HTR_{2A}$ is one of the most studied 5HT receptor since it's widely expressed in the cortex[232] and it is involved in hallucinogen actions[233]. On the other hand, $5HTR_{2C}$ is located principally in the VTA where it regulates negatively DA release to ACC[229].
$5HTR_3$ has been linked to fast excitatory and inhibitory neurotransmission; five different subtypes have been isolated in humans until now (A, B, D, E, F). Also this receptor is widely expressed throughout the brain and in particular in hippocampus, AMY, ACC, frontal and entorhinal cortex[234].
5HTR4 receptor has been involved in the long-term potentiation of hippocampus[235]; 5HTR4 KO animals show stress-induced feeding disorders[236].
5HTR5 has been less characterized: up to date, two different subtypes of the receptors are known (A, B) and both are localized in cortex and cerebellum[237]. Selective antagonists are able to impair memory[238] and reduce acoustic startle[239].
$5HTR_6$ and $5HTR_7$ are localized in thalamus, hypothalamus, hippocampus and peripheral tissues[229]; their function is still not clear even if $5HTR_6$ antagonists may have cognitive enhancing properties[232] and $5HTR_7$ ones antidepressant effect[240].

Figure 12 shows 5HT main pathways due to receptor binding.

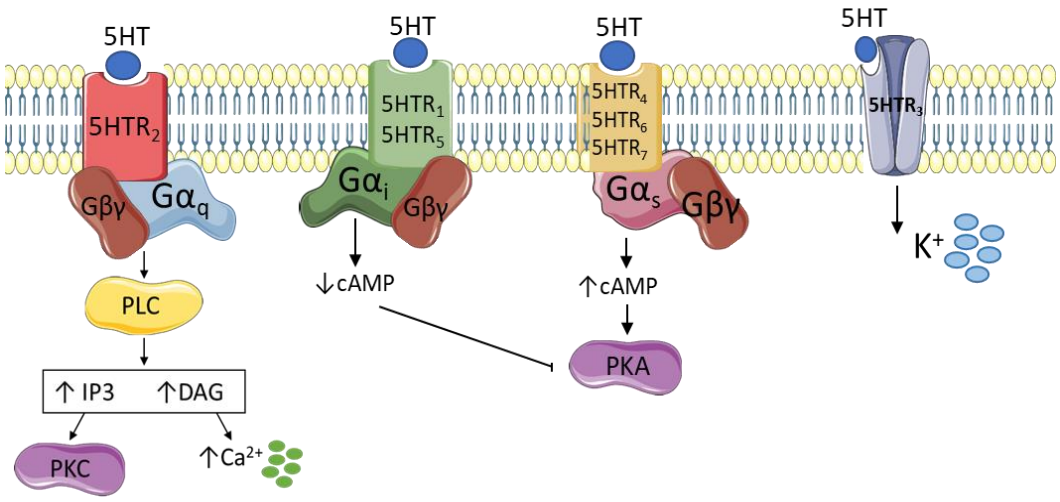

Figure 12: Serotonergic signalling pathways.

As said before, 5HT is a fundamental neurotransmitter regulating mood, behaviour, movement, pain appreciation, sexual activity, appetite, endocrine secretions, cardiac functions, sleep wake cycle. In the CNS, 5HT is synthetised in the raphe nuclei and in some parts of the brain stem. From here, fibres extend to many parts of the brain, including cerebral cortex and limbic areas, AMY, hypothalamus[215].

Various pathologies have been connected with serotoninergic system disruption: for example, excessive 5HT has been linked with anxiety, obsessive-compulsive disorders, anorexia, bulimia; while 5HT imbalances with NA are at the basis of depression[215].

Along with the other roles explicated by central 5HT, there's food intake. Direct action on neurons in ARC (a region of the brain that has strong importance in regulating feeding behaviour) is explicated by several serotonin drugs: for example, fenfluramine, a 5HT reuptake inhibitor and releasing agent, is able to stimulate POMC neurons firing[241]. In fact, both POMC and NPY-AgRP neurons in ARC present $5HTR_{1C}$ and $5HTR_{1B}$ respectively[222]. Also other cerebral regions may be involved in these actions, since 5HT neurons innervate also parabrachial nucleus and dorsal motor nucleus of the vagus and can act directly on the dopaminergic system through some GABA interneurons in the VTA presenting both $5HTR_{1A}$ and $5HTR_{2C}$[222]. Several studies have proven that drugs that increase the quantities of 5HT within synapses can cause wight reduction[242]. Actually, there are three mechanisms by

which 5HT is able to promote weight loss: it accelerates the onset of satiety[243], enhances basal metabolic rate[244] and inhibits carbohydrate craving[245]; that's why for long time drugs acting on the serotonergic system have been used as obesity treatments, even if with poor clinical results. The most interesting of these three actions, for the aim of this thesis, is 5HT role in carbohydrate craving. Animal studies suggest that eating carbohydrate-rich, protein-poor foods can act increasing tryptophan levels via the hormone insulin; thus enhancing TPH activity and 5HT production[246]. Confirming this hypothesis, rats given free access to carbohydrate-rich and protein-poor foods or normal chow, suppress the consumption of the former when administered with drugs that selectively rise 5HT release[247,248]. In addition, rise in the concentration of 5HT in response to carbohydrate consumption results in mitigating mood disturbances: there is a subset of obese patients whose weight problems are associated with atypical depression and uncontrolled carbohydrate intake[242]. In addition, peripheral 5HT is one of the main transmitters of gastrointestinal nervous system and can affect directly on digestion, energy intake, release of gastrointestinal hormones[249].

Clinical studies on obese people have detected various variations to the serotonergic system: in particular, a positive correlation between $5HTR_{2A}$ expression and BMI in cortical areas, hippocampus and insula; and increased $5HTR_4$ availability in multiple brain regions, including DLS[205]. Increased availability of 5HT receptors is linked to lower intrasynaptic 5HT concentration; supporting a hypo serotonergic hypothesis of obesity. Several studies have been investigating SERT variations in obese people, giving contradictory results and without finding a clear relationship between BMI and SERT alterations[205].

10. AIM OF THE STUDY

Obesity is becoming one of the most important health issues of our modern society[2], due to its spread diffusion and high number of associated comorbidities[14]. The high availability of caloric, energy dense food, rich in fats and sugar has led to the development of a new type of addiction, that is food addiction; where the consumption of high palatable food is necessary to alleviate negative states deriving from the mind[125]. Even if the relationship between obesity and mental health issues has been less investigated compared to other comorbidities, several epidemiologic studies suggest a link between obesity and depressive/anxiety disorders[142]. In particular, literature suggests that obesity is linked with neuroadaptive changes that provoke reduced reward response to food cues[112], thus diminishing the hedonical rewarding properties of food[208] and shifting food consumption from a positive reinforcement towards a negative one necessary to prevent or relieve mood alterations or somatic symptoms deriving from abstinence[137].

The aim of our study was to investigate whether exposure and subsequent abstinence from a cafeteria-style diet (rich in fat and sugar, based on the model of Johnson and Kenny[250]) was able to produce mood alterations and reward impairments in rats: behavioural tests were performed to assess the presence of anxiety-like behaviours and depressive-like phenotypes after 28 days of diet abstinence. Knowing the important role that monoamines have in the reward system and in the physiopathology of anxiety and depression[251], expression of enzymes responsible of monoamine synthesis (TH, DBH, TPH) along with DA, NA and 5HT concentration were analysed in key brain areas through immunofluorescence and HPLC analysis respectively. Along with monoamines, also endocannabinoid system role is fundamental in reward, mood and behaviour, food intake, inflammation and plasticity[134]. Therefore, western blot analysis of the expression of receptors and proteins of synthesis and degradation partaking to the endocannabinoid system were run in key brain areas, with the aim to evaluate its variations in diet exposure and consequent withdrawal in collaboration with prof. Fernando de Fonseca at the IBIMA institute of Malaga. Moreover, knowing that both obesity, anxiety and depression are pathologies associated with a chronic state of inflammation, we analysed the expression of GFAP and IBA-1, that are markers of neuroinflammation. With the aim to

correlate behavioural variations to neuroadaptive changes, several Pearson correlations tests were run to analyse the effect of diet exposure and abstinence: first of all, we analysed the relationship between behavioural parameters and monoamines, to discover which monoaminergic system is the most implied in mood alterations; then we run correlative analysis of single monoamine or protein within all the brain areas analysed, observing the functional interaction for each one throughout the different areas and lastly, we analysed the correlations between monoaminergic content and protein expression in each area, with the aim to study the interconnection between monoamines and endocannabinoid system in each brain region.

At the same time, second aim of our study was to evaluate if PF-3845 administration was able to revert the effects of diet exposure and withdrawal in reward, mood, inflammation, monoaminergic and endocannabinoid systems: as said in the introduction, PF-3845 is an irreversible inhibitor of FAAH and is able to rise the endogenous concentration of acylethanolamides and exert anxiolytic and antidepressant-like effect[252–254]. AEA, PEA and OEA have an important role in the brain, thanks to their binding to CB1 and PPAR-alpha receptor respectively: in particular, AEA controls emotionality and mood[167]; while OEA and PEA decrease appetite, reduce inflammation, participate to the control of reward related behaviours mediated by PPAR-alpha[168,255]. Several studies suggested that AEA, PEA and OEA have a role in the fine-tuning control of hedonic and homoeostatic aspect of food intake[255]; suggesting that their pharmacological increase might counteract the neuroadaptive changes that sustain the development of FA, obesity, and related comorbidities such as anxiety and depression.

Summarizing, our hypothesis was that cafeteria diet exposure and subsequent abstinence in rats might provoke neuroadaptive changes in the brain that turn into reward and behavioural alterations. At the same time, we imagined that the manipulation of the endogenous tone of acylethanolamides with PF-3845 might be effective in restoring reward and behavioural impairments through the modulation of brain monoaminergic and endocannabinoid system.

Seen so, main objectives of this thesis are:

- to investigate whether cafeteria diet exposure and abstinence can induce anxiety-like behaviour, depressive like phenotype, reward impairments, neuroinflammation and what neural changes in monoaminergic and endocannabinoid systems are associated with them;
- investigating whether PF-3845 administration can ameliorate such alterations and through which mechanism.

CHAPTER 2 – MATERIALS AND METHODS

1. ANIMALS, DIET AND EXPERIMENTAL DESIGN:

Male Wistar rats were purchased from Charles River; their body weight was around 300–350 g at the beginning of the experiments. Rats were group-housed under a 12 h light/dark cycle (lights on at 8:00 A.M.), at constant temperature (20 –22°C) and humidity (45–55%) and with access to food and water *ad libitum* for the entire period before starting the experiments. All experiments were performed in accordance with the European directive 2010/63/UE governing animal welfare and with the Italian Ministry of Health guidelines for the care and use of laboratory animals.

The first day of the experiment (day one), animals were randomly subdivided into two different groups:

- Control group (CHOW; n=14): rats were given *ad libitum* standard food pellets (4RF18; Mucedola; 2.6 kcal/g);
- Cafeteria group (CAF; n=12): rats were given *ad libitum* standard food and also extensive access (24h/7 days) to a CAFETERIA diet. Cafeteria diet model was developed in accordance with previous model described by Johnson PM, Kenny PJ[250] and consisted in exposing rats to a mixture of various foods available for human consumption: such as mortadella (3.2 kcal/g), cookies (Macine, 4.8 kcal/g) muffin (Mr Day 4.5 kcal/g), cheese chips (Fonzies; 5.3 kcal/g), cheese (4.3 kcal/g), sippets (San Carlo; 5.5 kcal/g) and lard (9 kcal/g). Each food was individually weighed before being made available to the rats. The caloric intake from the various foods was calculated based on the nutritional information provided by the manufacturer.

Animals followed the eating schedule described until the end of phase 1 of the experiment (from day 1 to day 40). At day 41, CAF rats underwent a 28 days abstinence period (phase 2 of the experiment) from CAFETERIA diet; in particular, CAF rats had only access *ad libitum* to standard food pellets (4RF18; Mucedola; 2.6 kcal/g)

until the end of the experiment (day 68). CHOW rats were maintained with *ad libitum* standard food until the end of the experiment.

At the same time, during phase 2 of the experiment (day 41-68), CAF rats were treated every other day with subcutaneous injections of PF-3845 10 mg/kg (CAF PF group; n=5) or with vehicle (VEH) consisting in ethanol/tween 80/saline in a proportion 5/5/90 v/v/v (CAF VEH group; n=7). Identically, also animals from CHOW group were treated either with PF-3845 10 mg/kg (CHOW PF group; n=6) or with VEH (CHOW VEH group; n=8). The experimental design is depicted in Fig.1.

Body-weight and food intake were measured every day, in both phase 1 and 2 of the experiment.

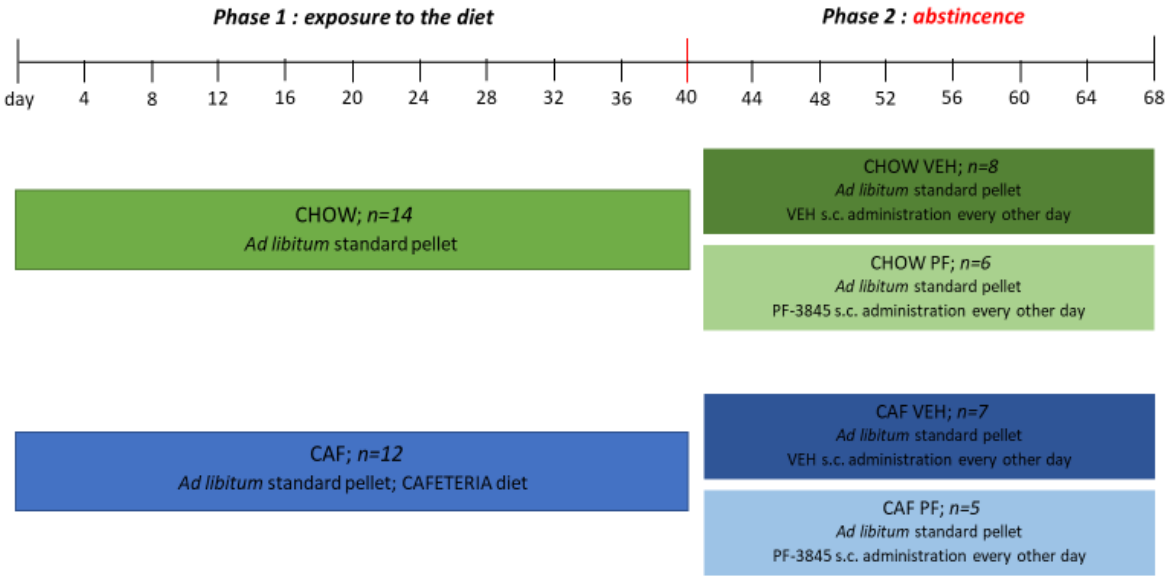

Fig.1: Experimental design. Rats were exposed to a cafeteria-style diet (including bacon, sausage, chocolate, cookies, etc) for 40 days. A control group of rats with ad libitum access only to standard chow and water was also included in the study. After the first 40 days of cafeteria diet exposure, rats underwent an abstinence period for 28 days, with no longer access to the cafeteria diet but still ad libitum access to standard chow. During the abstinence period, animals were treated either with the FAAH inhibitor PF-3845 (10 mg/kg, s.c.) or its vehicle administered every other day.

2. BEHAVIOURAL TESTS

At the end of the treatment rats underwent a battery of behavioural tests to evaluate whether cafeteria diet abstinence might have affected anxiogenic- and or depressive-like behaviour in our rat model and whether PF-3845 chronic treatment might rescue these effects.

In particular, on the day 68, 24h after the last administration of PF-3845 animals were subjected to elevated plus maze paradigm (EPM). The day after (first day without treatment) animals underwent open field test (OFT); day two and three without treatment rats were subjected to forced swimming test (FST). All behavioural test were carried in a sound-attenuated room illuminated by a dim red light (30 lux). Figure 2 shows the timeline of behavioural tests.

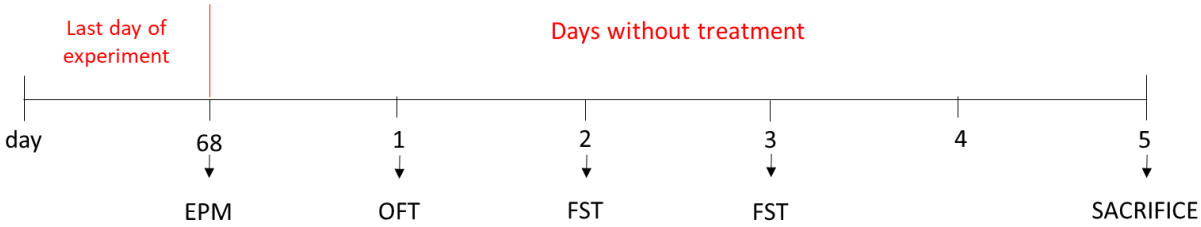

Fig.2: Timeline of behavioural tests.

2.1 OPEN FIELD TEST (OFT):

OFT is a behavioural test used for the evaluation of the general locomotor activity of rats which is strictly related to their feeling of anxiety. It was developed by the American psychologist Calvin S. Hall in 1932 to test the emotional behaviour of rats. Lots of variables can be examined during the OFT but the most important are the thigmotaxis, which is the propensity of the rat to stay near the walls of the arena (index of an anxiety-like behaviour) and habituation, evaluated as exploratory behaviour, ongoing in a novel environment[256].

For the execution of OFT, automated locomotor activity boxes (square plastic boxes with a 43 x 43 cm arena and a 25 x 25 cm central zone; Med Associates, St Albans, Vermont, USA) were used to quantify spontaneous activity parameters. Locomotor activity was recorded automatically by interruption of two orthogonal light beams (3.5

and 13 cm above the activity box floor), which were connected to automatic software. Increased locomotor activity in the entire field was considered a sign of behavioural arousal, while a reduced locomotor activity in the central zone and numbers of entries into the central zone were considered signs of increased emotionality, anxiety, or fear in rodents. Each animal was tested for twenty minutes. Between test sessions, the apparatus was cleaned with alcohol (70%) and dried with a cloth.

2.2 ELEVATED PLUS MAZE PARADIGM (EPM):

The elevated plus-maze (EPM) test aims to detect anxiety behaviour in rodents reflected by avoidance of open area exploration[257]. It was developed by the researcher Jon K. Sheperd; who noticed that rats in an open, closed or elevated space display a conflict between the natural explorative behaviour and the aversion for open/elevated spaces. Animals obliged to stay in open arms display fear, immobility, defecation, an increment of plasma corticosteroids. Similarly to the OFT, animals with anxiety-like behaviour prefer staying in closed arms and avoid the open ones[258].

The EPM used for our experiments consisted of two open arms and two closed arms each measuring 55 (length) × 10 cm (width), with black wooden floors emanating from a common central platform (10×10 cm) to form a plus shape. The open arms were without edges both on the sides and ends, while the closed arms had 40-cm high painted black wood walls. The maze was elevated 80 cm above the ground. The rats were brought to the room in a clear plastic neutral box measuring 32×22×11 cm. The maze was carefully cleaned between each rat exposure and positioned in a closed white room. Parameters measured included time spent in the closed arms, in the open arms and in the centre. The animals were placed in the centre of the maze facing an open arm. They were observed and scored via a closed-circuit TV camera fixed at the ceiling with an observation monitor located in an adjacent room and their behaviour recorded by an expert group of experimenters, unaware of the treatments, for a period of 5 min.

2.3 FORCED SWIMMING TEST (FST):

FST is known also as Porsolt FST; from the name of the behavioural scientist who invented it in 1977. The method was based on the observation that the rat, when forced to swim without possibility to escape, stops moving completely after the initial period of intense activity (swimming, climbing) and performs only the movements necessary to keep the head above water. This state of easily identifiable behavioural immobility has been described as the state of "despair" when the animal realizes that the escape is impossible and gives up[256]. FST is the most widely used test to evaluate the effects of antidepressants. Interestingly, antidepressants drugs reduce the immobility time, which is used as the main predictor of antidepressant action[256].

In our study, each rat was put in a plexiglass cylinder (diameter 29 cm; height 50 cm) filled with water at 25-27 °C of temperature. Each animal was subjected to two swimming sessions separated by 24 h: the first session lasted 15 min, whereas the second one, the only monitored, 5 min. Water was changed after every trial and animals were gently dried with a towel after swimming.

3. SACRIFICE:

Two days after the last behavioral test (fifth day without treatment), the animals were euthanized by CO_2 overdose. Brains were extracted and immediately snap-frozen in 2-methyl butane (-50°C) and stored at -80°C until processed.

4. CEREBRAL DISSECTION:

Each brain was cut with a cryostat (model HM550; Thermo Fisher Scientific, Kalamazoo, MI, USA) partially in 20 µm coronal sections mounted on positively charged microscope slides (SuperFrost Plus, Menzel, Germany) and stored at −80 °C for immunofluorescence analysis and partially microdissected into different regions of interest collected in microtubes (pooled from both hemispheres), weighed to a high degree of accuracy by using a microbalance and stored at -80°C until processed.

In particular, we collected from both hemispheres: the prefrontal cortex (PFC), medial prefrontal cortex (mPFC), dorsolateral striatum (DLS), accumbens (ACC), amygdala (AMY), hypothalamus (HYPO), ventral pallidum (VPL), dorsal hippocampus (dHIPPO), ventral hippocampus (vHIPPO), periaqueductal grey (PAG), dorsal raphe (DR), locus coeruleus (LC), ventral tegmental area (VTA), substantia nigra (SN), lateral parabrachial (LPB).

Figures 3-8 show significative images from Paxino's brain atlas[259], used as a reference while micro punching and dissecting areas.

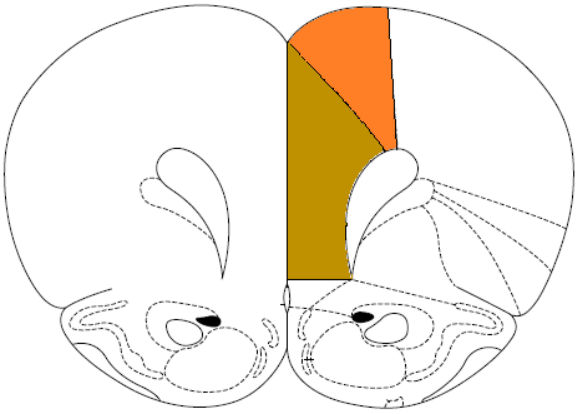

Fig.3: Representative image of figure 8 from Paxino's rat brain atlas. Dissected regions of interest in this area were mPFC (in orange) and PFC (in brown).

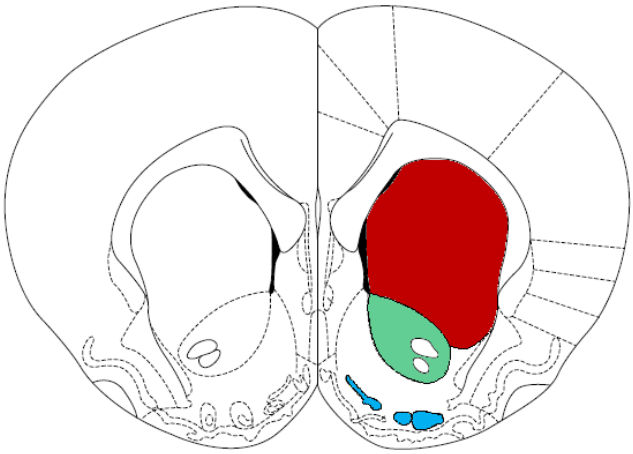

Fig.4: Representative image of figure 11 from Paxino's rat brain atlas. Dissected regions of interest in this area were DLS (in red); ACC (in green); VPL (in light blue).

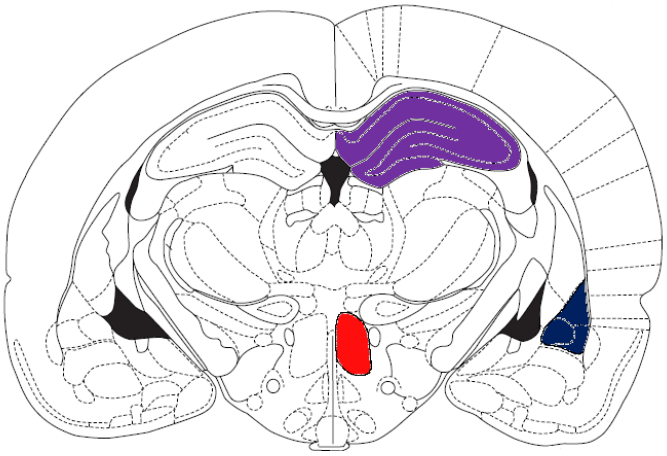

Fig.5: Representative image of figure 35 from Paxino's rat brain atlas. Dissected regions of interest in this area were DHIPPO (in violet); HYPO (in red); AMY (in blue).

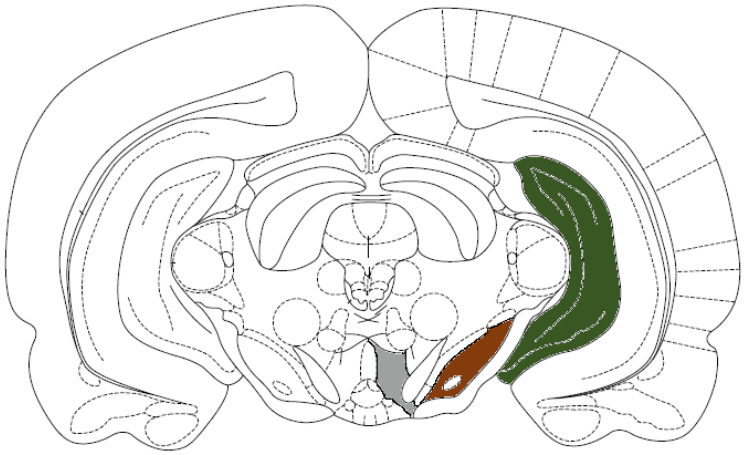

Fig.6: Representative image of figure 44 from Paxino's rat brain atlas. Dissected regions of interest in this area were VHIPPO (in green); SN (in brown); VTA (in grey).

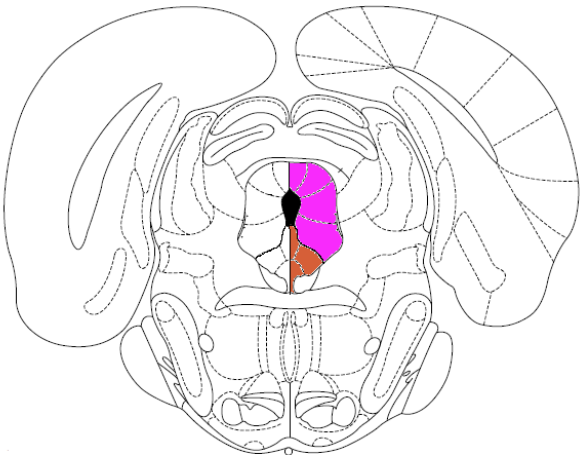

Fig.7: Representative image of figure 51 from Paxino's rat brain atlas. Dissected regions of interest in this area were PAG (in pink); DR (in brown).

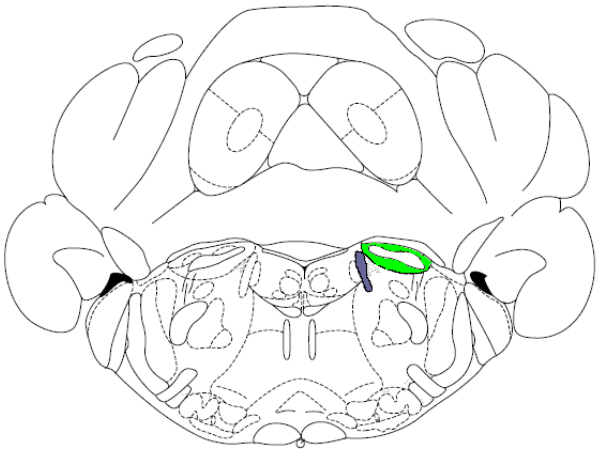

Fig.8: Representative image of figure 58 from Paxino's rat brain atlas. Dissected regions of interest in this area were LC (in grey); LPB (in green).

5. IMMUNOFLUORESCENCE ANALYSIS:

5.1 TH and CB1 double fluorescence immunostaining

TH and CB1 double fluorescence immunostaining was performed on brain section series containing PAG, DR, SN, VTA, DLS, ACC. Sections were defrosted and post-fixed in a 4% PFA solution for 15 minutes at 4°C. Then, sections were rinsed with PB (0.1 M; pH= 7.4) and incubated for 1 hour with a solution containing 0.3% Triton X-100 *(Sigma Aldrich)*, 1,5% Normal Goat Serum *(Jackson Immunoresearch)*, 1% Normal Donkey Serum *(Jackson Immunoresearch)*. After additional washes, sections were incubated with primary antibody solution containing mouse anti-TH antibody (1/900; LNC1, *Millipore*) and CB1 rabbit antiserum 2825.3 (raised against C-terminal residues 461–473) for 2 overnights at 4°C. Sections were then incubated with Donkey anti-mouse Alexa Fluor 488 secondary antibody (1/250 dilution, *Invitrogen*) and Goat anti-rabbit Alexa Fluor 594 secondary antibody (1/400, *Invitrogen*) for 90 minutes in the presence of Hoechst 33258 (1:5000 dilution; *Sigma–Aldrich*), used to detect cell nuclei. After final washes slides were cover-slipped with Fluoromount (*Sigma Aldrich*).

5.2 DBH and CB1 double fluorescence immunostaining

DBH and CB1 double fluorescence immunostaining was performed on brain section series containing LC. Sections were defrosted and post-fixed in a 4% PFA solution for

15 minutes at 4°C. Then, sections were rinsed with PB (0.1 M; pH= 7.4) and incubated for 1 hour with a solution containing 0.3% Triton X-100 *(Sigma Aldrich)*, 2% Normal Goat Serum *(Jackson Immunoresearch)*, 1.5% Normal Donkey Serum *(Jackson Immunoresearch)*, 1.5% BSA (*Serva Electrophoresis*). After additional washes, sections were incubated with primary antibody solution containing mouse anti-DBH antibody (1/900; MAB308, *Millipore*) and CB1 rabbit antiserum 2825.3 (raised against C-terminal residues 461–473) for 2 overnights at 4°C. Sections were then incubated with Donkey anti-mouse Alexa Fluor 488 secondary antibody (1/350 dilution, *Invitrogen*) and Goat anti-rabbit Alexa Fluor 594 secondary antibody (1/400, *Invitrogen*) for 90 minutes in the presence of Hoechst 33258 (1:5000 dilution; *Sigma–Aldrich*), used to detect cell nuclei. After final washes slides were cover-slipped with Fluoromount (*Sigma Aldrich*).

5.3 TPH fluorescence immunostaining

TPH fluorescence immunostaining was performed on brain section series containing DR. Sections were defrosted and post-fixed in a 4% PFA solution for 15 minutes at 4°C. Then, sections were rinsed with PB (0.1 M; pH= 7.4) and incubated for 1 hour with a solution containing 0.3% Triton X-100 *(Sigma Aldrich)*, 2% Normal Donkey Serum *(Jackson Immunoresearch)*, 1.5% BSA (*Serva Electrophoresis*). After additional washes, sections were incubated with primary antibody solution containing mouse anti-TPH antibody (1/500; WH-3, *Sigma Aldrich*) overnight at 4°C. Sections were then incubated with Donkey anti-mouse Alexa Fluor 488 secondary antibody (1/250 dilution, *Invitrogen*) for 90 minutes in the presence of Hoechst 33258 (1:5000 dilution; *Sigma–Aldrich*), used to detect cell nuclei. After final washes slides were cover-slipped with Fluoromount (*Sigma Aldrich*).

5.4 Image acquisition and quantification

All brain sections included in the analyses were observed under a Nikon Eclipse 80i microscope equipped with a color charge-coupled device camera and controlled by the software NIS-Elements-BR (Nikon). Slices were photographed under epifluorescent conditions. The rat brain atlas by Paxinos and Watson was used as reference for the localization of the brain areas of interest. Immunostaining was measured semi-

quantitatively as optical density (OD) by using the ImageJ software and considering, for background normalization, the averaged OD either of non-immunoreactive regions or of white matter structures within the same brain slice. The investigator was blind to animal treatment; measurements were obtained in at least five consecutive tissues sections per animal containing the desired structure.

6. HPLC ANALYSIS

For the HPLC analysis; all the sections form the right hemisphere were analysed. Samples were ultrasonicated in ice-cold 0.1 M perchloric acid and then centrifuged at 15000g for 20 min at 4°C as described by Cassano et al[260]. Supernatants were collected and used for monoamines and monoamine metabolites assay. Sample of selected brain regions of all groups of animals were run in parallel to eliminate the possibility of run effects. Tissue levels of monoamines (dopamine or DA, noradrenaline or NA, serotonin or 5HT) and both 5HT and DA metabolites were detected and quantified by HPLC. Monoamines and their metabolites were analyzed by microbore HPLC, the detection was accomplished with a Unijet cell (BAS) with a 6-mm diameter glassy carbon electrode set at +650 mV vs an Ag/AgCl reference electrode, connected to an electrochemical amperometric detector (INTRO, Antec Leyden, Netherlands). The analytes were separated using a SphereClone 150-mm × 2-mm column (3-µm packing) and a mobile phase composed of 85 mM of sodium acetate, 0.34 mM EDTA, 15 mM sodium chloride, 0.81 mM of octanesulphonic acid sodium salt, 6% methanol (v/v) (pH = 4.85) delivered at a flow rate of 800 µl/min for a total runtime of 35 min. For each analysis, a set of standards containing various concentrations of each compound (monoamines and their metabolites) was prepared in the acid solution to obtain appropriate calibration curves. The concentrations of neurotransmitters were determined by linear interpolation from standard curves; we normalized their concentration to the weight of the wet tissue sample. DA and 5-HT turnover were calculated as the ratio between the metabolite and the monoamine (DOPAC+HVA/DA for the DA and 5HIAA/5HT for the 5HT).

7. WESTERN BLOT ANALYSIS

For western blot analysis (WB); all the sections form the left hemisphere were analysed. Protein extraction was performed in RIPA buffer (Tris HCl 50 mM, NaCl 150 mM, NP40 1%, Na DOC 0,25%, EDTA 1 mM; pH 7.4) with a cocktail of protease and phosphatase inhibitors (PMS 1mM, Pepstatina 1 µg/mL, Leupeptina 5 µg/mL, Aprotinina 5 µg/mL, T.I. 10 µg/mL, $NaVO_4$ 1 mM, NaF 1 mM) added in proportion to the weight of each sample. Samples were homogenized in a tissue lizer (TissueLizer 2 QUIAGEN) for three minutes at 20 Hz and then centrifuged (15 minutes, 12000 g, 4°C). The supernatant was collected, analysed with BRADFORD essay and then each sample was added with DTT in proportion to the quantity of protein. Extracted proteins were stored at -20°C until blotting.

30µL of each sample were run in a gel Criterion XT Precast Gel 4-12% Bis-Tris acrylamide in MOPS (BIORAD) at 130 V. Proteins were transferred to a nitrocellulose membrane; then the membrane was blocked for one hour in BSA 2% and incubated overnight with the corresponding primary antibody [TABLE 1]. Membranes were rinsed and incubated with secondary antibody [TABLE 2], then revealed with LUMINOL ECL PRIME WESTERN BLOTTING DET (GE HEALTHCARE) in a CHEMIDOC (BIORAD). Each primary antibody detected a protein of the expected molecular size. The protein intensity was quantified with the image processing software ImageJ (Rasband, W.S., ImageJ, U.S., NIH, http://imagej.nih.gov/ij, 1997–2012). The results were expressed as the protein/adaptin ratio.

PRIMARY ANTIBODY	MANIFACTURING DETAILS:	DILUITION
Mouse anti- adaptina	Biorbyt	1/2000
Rabbit anti- CB1	Abcam	1/200
Rabbit anti- CB2	Abcam	1/200
Rabbit anti- NAPE PLD	Abcam	1/1000
Rabbit anti- FAAH	Cayman	1/100
Rabbit anti- DAGLbeta	Biorbyt	1/100
Rabbit anti- DAGLalpha	Biorbyt	1/100
Rabbit anti- MAGL	Frontier Institute	1/200
Rabbit anti- COX2	CellSignaling	1/500
Rabbit anti- CX3CR1	Abcam	1/500
Rabbit anti- IBA1	Wako	1/500
Mouse anti- GFAP	Sigma Aldrich	1/400

Table 1: List of all the primary antibodies used for WB analysis; with its manufacturer and dilution.

SECONDARY ANTIBODY	MANIFACTURING DETAILS:	DILUITION
Goat anti-mouse	Promega	1/10000
Goat anti-rabbit	Promega	1/10000

Table 2: List of all the secondary antibodies used for WB analysis; with its manufacturer and dilution.

8. STATISTICAL ANALYSIS:

All data shown in this thesis are expressed as MEAN±SEM. The data obtained from the daily monitoring of the body weight gain and food intake in phase one were analysed by a two-way ANOVA for repeated measures, setting "diet" (CHOW, CAF) and "time" as fixed variables, and the Bonferroni's test was used as post-hoc analysis for multiple comparisons.

Similarly, data from phase two were analysed with a two-way ANOVA for repeated measures (within the same treatment group), setting "diet" (CHOW, CAF) and "time" as fixed variables; the Bonferroni's test was used as post-hoc analysis for multiple comparisons and with a Two-way ANOVA for repeated measures (within the same diet group), setting "treatment" (VEH, PF-3845) and "time" as fixed variables, the Bonferroni's test was used as post-hoc analysis for multiple comparisons.

On the other hand, data obtained from the behavioural test, immunofluorescence, HPLC and WB analysis were analysed by Two-way ANOVA, with "diet" (CHOW, CAF) and "treatment" (VEH, PF-3845) as fixed variables, and Tukey's test was used as post-hoc for multiple comparisons. Moreover, for immunofluorescence analysis and western blot analysis, because of the difference in the number of samples examined and the high degree of freedom, the error degrees of freedom were kept constant at a value based on the actual number of animals per group used in each experiment.

Two-tail Bravais – Pearson correlations tests were performed for each experimental group to correlate different parameters. Only correlations with $0.7<r<1$ and $-1<r<-0.7$ were considered. All the Two-way ANOVA analyses and the Pearson's correlation tests were carried out using SPSS Statistics (IBM Corporation, Armonk, NY, USA), while Bonferroni post-hoc's were performed using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). In all instances, the significance threshold was set at $P<0.05$.

CHAPTER 3 – RESULTS

1. BODY WEIGHT AND FOOD INTAKE

Phase 1:

The first phase of the experiment (days 1-40) was aimed to induce obesity in rats by hedonic overfeeding; thanks to the exposure to a CAFETERIA diet, as explained in the materials and methods paragraph.

As expected, the exposure to CAFETERIA diet regimen caused rats to gain weight: since day 10 on, there was a significant difference between the weight of CAF group compared to CHOW one. Two-way ANOVA for repeated measures analysis of body weight of phase 1 showed a significant effect of diet regimen *(F_{diet}= 24,536, df=20, p<0,001)*, a significant effect of time *(F_{time}= 381,917, df=20, p<0,001)*; moreover interaction between the two factors was observed (*$F_{interaction}$= 46,037, df=20, p<0,001*). Bonferroni's post-hoc analysis results are shown in figure 1.

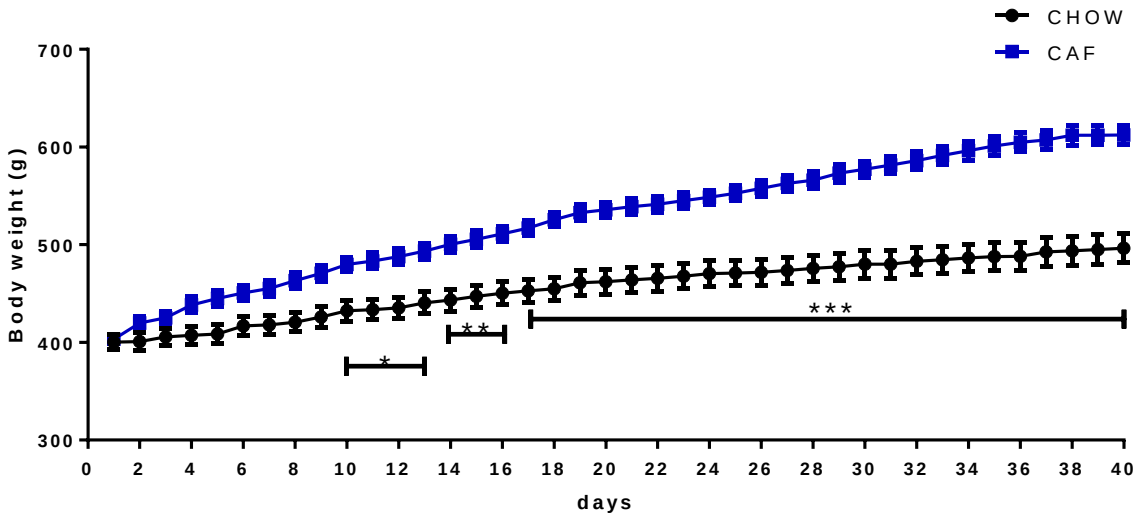

Fig.1: Time course of body weight (g) during phase 1 of the experiment. Bodyweight was monitored every day. Results are expressed as MEAN±SEM. *p<0,05; **p<0,01; ***p<0,001 in the same day (Bonferroni's post-hoc test for between-groups comparisons). CHOW group n=11; CAF group n=10.

Significant body weight gain variation between the two groups was caused by significant variations of food intake: the analysis of daily caloric intake showed that animals exposed to CAFETERIA diet significantly consumed more calories compared to CHOW group. In fact, two-way ANOVA for repeated measures analysis of normalized food intake of phase 1 showed a significant effect of diet regimen *(F_{diet}= 209,427, df=20, p<0,001)*, a significant effect of time *(F_{time}= 5,484, df=20, p<0,001)*; moreover interaction between the two factors was observed (*$F_{interaction}$= 1,966, df=20, p<0,001*). Bonferroni's post-hoc analysis results are shown in figure 2.

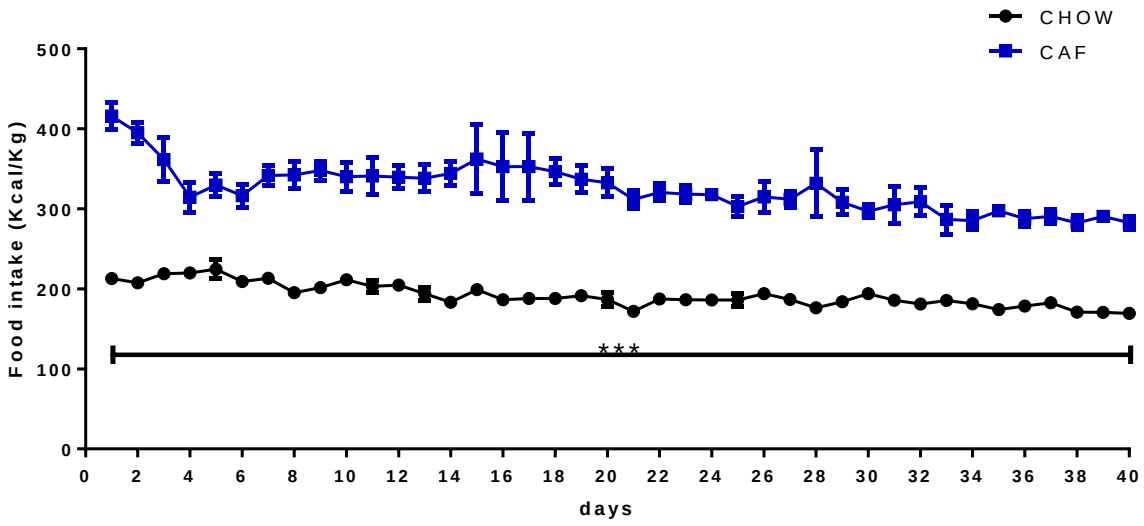

Fig.2: Time course of food intake (Kcal/Kg) during phase 1 of the experiment. Food intake was monitored every day. Results are expressed as MEAN±SEM. ***p<0,001 on the same day (Bonferroni's post-hoc test for between-groups comparisons). CHOW group n=11; CAF group n=10.

Phase 2:

During phase 2 of the experiment (days 41-68) rats experienced abstinence from CAFETERIA diet. As expected, the withdrawal from CAFETERIA diet caused rats to reduce both their food intake and their body weight in a significant manner: CAF rats, after being used to consume high palatable food reduced their caloric intake in the first twelve days of phase two as part of their physiological counter-response (days 41-

53). After this first period of adaptation, rats began to consume the same amount of food of the CHOW group, which was never exposed to CAFETERIA diet and, by the end of the experiment, no significant difference was detected in the body weight or in the food intake of animals from the different groups. Moreover, it is important to notice that the administration of PF-3845 was unable to affect the body weight and food intake both in CAF and CHOW animals.

In fact, the two-way ANOVA for repeated measures of body weight of phase 2 between the groups CHOW VEH and CAF VEH showed a significant effect of diet regimen *(F_{diet}= 9,927, df=8, p<0,05)*, a significant effect of time *(F_{time}= 4,143, df=8, p<0,001)*; moreover interaction between the two factors was observed (*$F_{interaction}$= 41,967, df=8, p<0,001*). Differently, the two-way ANOVA between the groups CHOW PF and CAF PF did not show a significant effect of the diet *(F_{diet}= 1045,712, df=9, p>0,05*), but a significant effect of time *(F_{time}= 9,320, df=9, p<0,001)*; interaction between the two factors was observed (*$F_{interaction}$= 25,194, df=9, p<0,001*). The same analysis for the body weight between the groups CHOW VEH and CHOW PF did not display a significant effect of the treatment *($F_{treatment}$= 867,323, df=9, p>0,05)*, but a significant effect of time *(F_{time}= 29,324, df=9, p<0,001)*; moreover interaction between the two factors was observed (*$F_{interaction}$= 5,394, df=9, p<0,001*). At the same time, the two-way ANOVA for repeated measures between the groups CAF VEH and CAF PF did not show a significant effect of the treatment *($F_{treatment}$= 0,345, df=8, p>0,05)*, but a significant effect of time *(F_{time}= 38,228, df=8, p<0,001)*; no interaction between the two factors was observed (*$F_{interaction}$= 0,394, df=8, p>0,05*). Bonferroni's post-hoc analysis results are shown in figure 3.

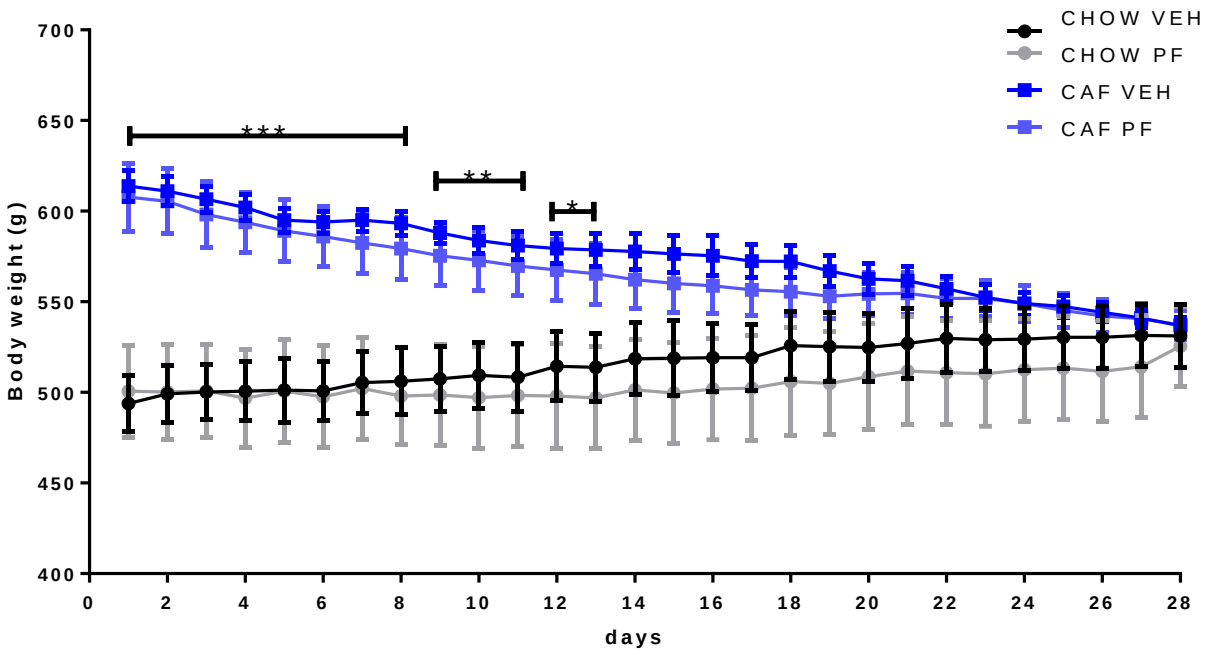

Fig.3: Time course of body weight (g) during phase 2 of the experiment. Bodyweight was monitored every day. Results are expressed as MEAN±SEM. *p<0,05; **p<0,01; ***p<0,001 in the same day between CHOW VEH and CAF VEH animals (Bonferroni's post-hoc test for between-groups comparisons). CHOW VEH group n=5; CHOW PF group n=6; CAF VEH group n=5; CAF PF group n=5.

On the other hand, in the analysis of food intake, the two-way ANOVA for repeated measures of phase 2 between the groups CHOW VEH and CAF VEH showed a significant effect of diet regimen *(F_{diet}=16,872, df=8, p<0,01)*, a significant effect of time *(F_{time}=10,510, df=8, p<0,001)*; moreover interaction between the two factors was observed (*$F_{interaction}$=11,550, df=8, p<0,001*). The two-way ANOVA between the groups CHOW PF and CAF PF displayed a significant effect of the diet *(F_{diet}=31,773, df=9, p<0,001*), and a significant effect of time *(F_{time}= 24,147, df=9, p<0,001)*; interaction between the two factors was observed (*$F_{interaction}$= 27,998, df=9, p<0,001*). The same analysis for the food intake between the groups CHOW VEH and CHOW PF did not display a significant effect of the treatment *($F_{treatment}$= 0,342, df=9, p>0,05)*, but a significant effect of time *(F_{time}= 1,546, df=9, p<0,05)*; moreover interaction between the two factors was observed (*$F_{interaction}$= 1,812, df=9, p<0,05*). At the same time, the two-way ANOVA for repeated measures between the groups CAF VEH and CAF PF did not show a significant effect of the treatment *($F_{treatment}$= 4,372, df=8, p>0,05)*, but a significant effect of time *(F_{time}= 73,804, df=8, p<0,001)*; interaction between the two

factors was observed ($F_{interaction}$= *4,183, df=8, p<0,001*). Bonferroni's post-hoc analysis results are shown in figure 4.

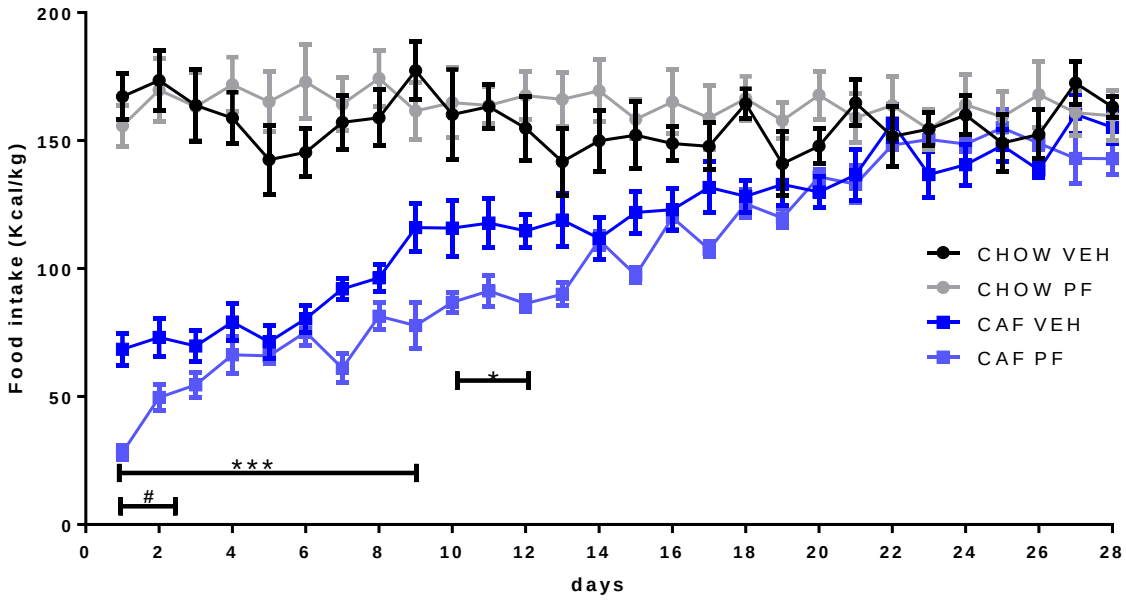

Fig.4: Time course of food intake (kcal/kg) during phase 2 of the experiment. Food intake was monitored every day. Results are expressed as MEAN±SEM. *p<0,05; ***p<0,001 in the same day between CHOW VEH and CAF VEH animals; #p<0,05 in the same day between CHOW PF and CAF PF animals (Bonferroni's post-hoc test for between-groups comparisons). CHOW VEH group n=5; CHOW PF group n=6; CAF VEH group n=5; CAF PF group n=5.

2. BEHAVIOURAL TESTS:

Behavioural tests were performed to elucidate whether the long-term withdrawal from CAFETERIA diet was able to induce anxiogenic-like behaviour or a depressive-like phenotype in rats.

It is well known that the withdrawal from the consumption of tobacco[261] or alcohol[262] can cause anxiety and depression. Our study in this phase aims to elucidate whether also the withdrawal from the consumption of high-caloric high-palatable food as the one consumed by rats in CAFETERIA diet could cause anxiety and depression; considering that several studies demonstrate that the withdrawal of the palatable diet can lead to a stress-like response[10].

2.1 OPEN FIELD TEST (OFT):

In the OFT we evaluated different parameters: the total distance travelled in the arena, the zone entries in the centre of the arena and the distance travelled in the centre of the arena.

Animals abstinent from the CAF diet did not show a significant reduction of the total travelled distance. However, CAF PF group showed a significant reduction of the total distance travelled when compared to CHOW PF one.

Two-way ANOVA for the total distance travelled revealed a significant effect for the diet *(F_{diet}=6,435, df=20, p<0,05*); while there was no significant effect for treatment (*$F_{treatment}$= 2,762, df=20, p=0,115*) neither significant interaction between the two factors (*$F_{interaction}$= 0,460, df=20, p=0,507*). Tukey's post hoc analysis results are shown in figure 5.

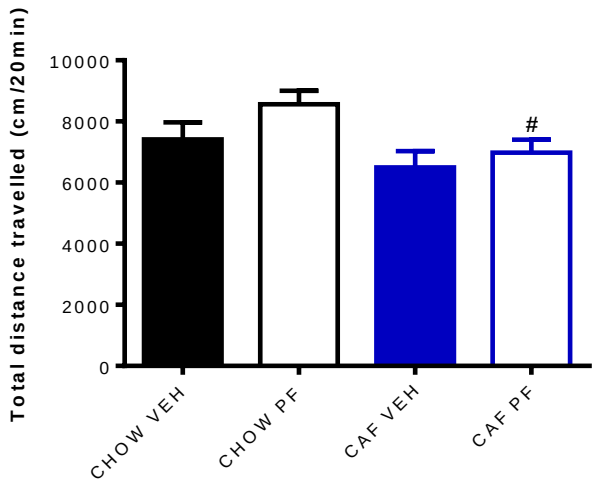

Fig.5: Total distance travelled during the OFT expressed in cm/20 min. Results are expressed as MEAN±SEM. #p<0,05 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=5; CHOW PF group n=6; CAF VEH group n=5; CAF PF group n=5.

Differently, CAF VEH animals showed a significant decrease of the zone entries in the central part of the arena compared to the control group; suggesting that they were developing an anxiogenic-like behaviour. In this case, the pharmacological treatment with PF-3845 in CAF PF group was significantly able to increase the zone entries in the centre of the arena compared to CAF VEH ones; suggesting its ability to have an anxiolytic-like effect.

Two-way ANOVA for the number of zone entries revealed a significant effect for the diet *(F_{diet}=6,613, df=20, p<0,05*); and treatment (*$F_{treatment}$= 4,635, df=20, p<0,05*); there was significant interaction between the two factors (*$F_{interaction}$= 7,378, df=20, p<0,05*). Tukey's post hoc analysis results are shown in figure 6.

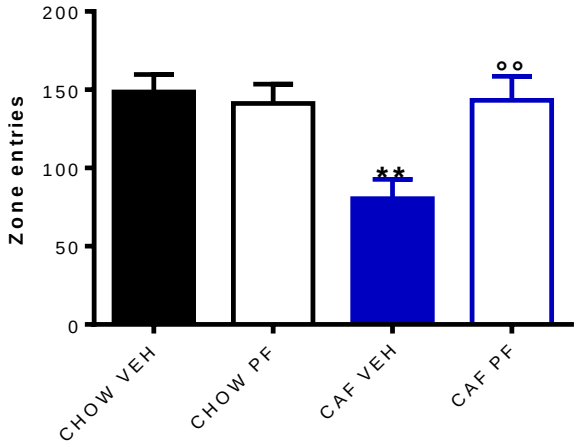

Fig.6: Total number of the zone entries during the OFT. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; °°p<0,01 vs CAF VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=5; CHOW PF group n=6; CAF VEH group n=5; CAF PF group n=5.

Distance travelled in the centre of the arena follows the same trend of the zone entries: CAF VEH animals displayed a significant reduction of the centre distance travelled compared to CHOW VEH ones. PF-3845 treatment was effective in CAF PF animals in raising the centre distance travelled when compared to CAF VEH ones; exerting an anxiolytic-like effect.

Two-way ANOVA for the total distance travelled in the centre revealed significant effect for the diet *(F_{diet}=8,217, df=20, p<0,05*) and for the treatment (*$F_{treatment}$= 5,536, df=20, p<0,05*); significant interaction between the two factors was detected (*$F_{interaction}$= 4,510, df=20, p<0,05*).

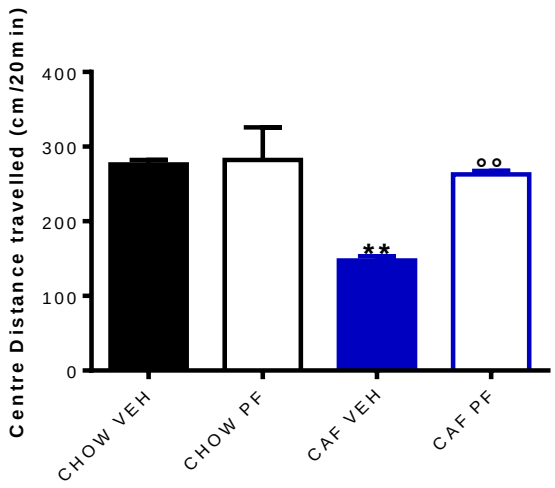

Fig.7: Total distance run in the centre of the arena by the animals during OFT expressed in cm/20min. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; °°p<0,01 vs CAF VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=5; CHOW PF group n=6; CAF VEH group n=5; CAF PF group n=5.

2.2 ELEVATED PLUS MAZE (EPM):

In the EPM paradigm, we analysed the time spent by the animals in closed arms; the centre of the maze; open arms and both in the centre and open arms.

Analysis of these data revealed that diet withdrawal was significantly able to increase the time spent in closed arms and decrease the time spent in open arms and in the centre and open arms for CAF VEH animals when compared to CHOW VEH group. Consequently, we can affirm that these animals displayed an anxiogenic-like behaviour; considering their aversion for the open spaces and preference for the closed ones; confirming the data from OFT.

Also, in this case, the pharmacologic treatment with PF-3845 in CAF PF animals was significantly able to decrease the time spent in the closed arms and increase the one spent in open arms and in centre and open arms when compared to vehicle-treated ones; confirming its anxiolytic effect observed also in the OFT.

Two-way ANOVA for the time spent in closed arms revealed significant effect for the diet (F_{diet}*=5,459, df=20, p<0,05*); for treatment ($F_{treatment}$*= 4,685, df=20, p<0,05*); interaction between the two factors was observed ($F_{interaction}$*= 7,155, df=20, p<0,05*). Tukey's post hoc analysis results are shown in figure 8.

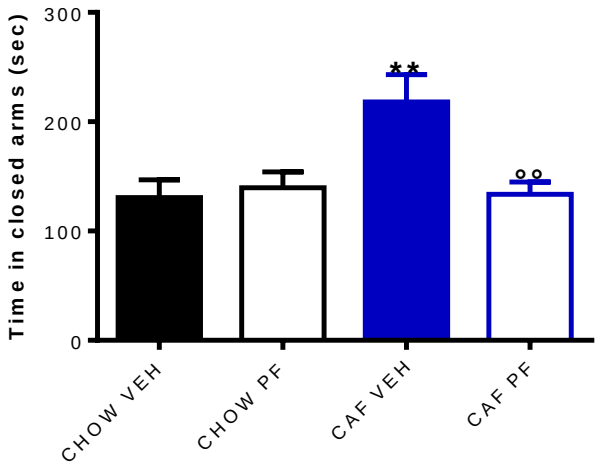

Fig.8: Time in seconds spent in closed arms during the EPM paradigm. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; °°p<0,01 vs CAF VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=5; CHOW PF group n=6; CAF VEH group n=5; CAF PF group n=5.

Two-way ANOVA for the time spent in open arms revealed significant effect for the diet *(F_{diet}=9,052, df=20, p<0,01*) and for treatment (*$F_{treatment}$= 4,822, df=20, p<0,05*); significant interaction between the two factors was observed (*$F_{interaction}$= 4,668, df=20, p<0,05*).

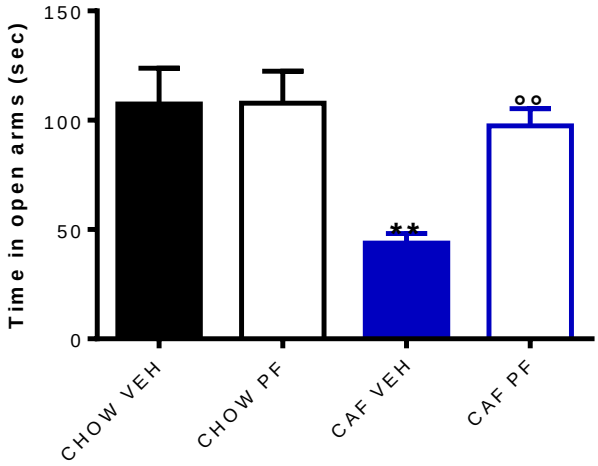

Fig.9: Time in seconds spent in open arms during the EPM paradigm. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; °°p<0,01 vs CAF VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=5; CHOW PF group n=6; CAF VEH group n=5; CAF PF group n=5.

Two-way ANOVA for the time spent in the centre of the maze revealed no significant effect for the diet *(F_{diet}=0,071, df=20, p=0,793*) and for treatment (*$F_{treatment}$= 0,586, df=20, p=0,455*) neither significant interaction between the two factors (*$F_{interaction}$= 2,048, df=20, p=0,171)*.

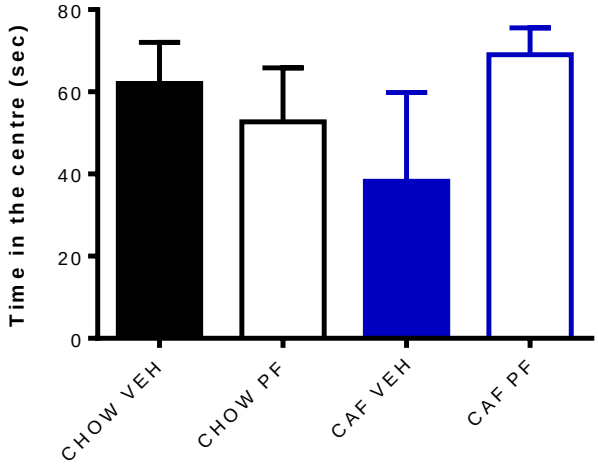

Fig.9: Time in seconds spent in the centre of the maze during the EPM paradigm. Results are expressed as MEAN±SEM. CHOW VEH group n=5; CHOW PF group n=6; CAF VEH group n=5; CAF PF group n=5.

Two-way ANOVA for the time spent in the centre of the maze and in open arms revealed significant effect for the diet *(F_{diet}=5,459, df=20, p<0,05*) and treatment (*$F_{treatment}$= 4,685, df= 1/20, p<0,05*); there was significant interaction between the two factors (*$F_{interaction}$= 7,155, df= 1/20, p<0,05*). Tukey's post-hoc analysis results are shown in figure 10.

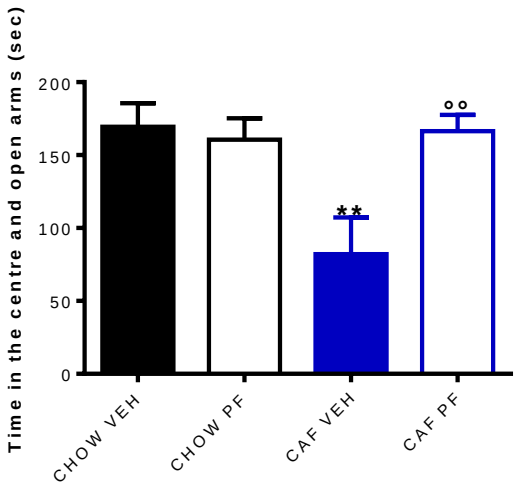

Fig.10: Time in seconds spent in the centre of the maze and open arms during the EPM paradigm. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; °°p<0,01 vs CAF VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=5; CHOW PF group n=6; CAF VEH group n=5; CAF PF group n=5.

2.3 FORCED SWIMMING TEST (FST):

In the FST, we evaluated the immobility time of rats. We observed a significant increase of the immobility time of the animals abstinent from the diet compared to control group, suggesting that abstinence from the diet could cause the development of a depressive-like phenotype. Pharmacological treatment with PF-3845 in CAF PF rats was significantly able to reduce the immobility time when compared to CAF VEH ones; exerting an anti-depressant like effect.

Two-way ANOVA for the immobility time revealed no significant effect for the diet (F_{diet}*=1,304, df=20, p=0,269*) but a significant effect for treatment ($F_{treatment}$*= 6,879, df=20, p<0,05*); there was a significant interaction between the two factors ($F_{interaction}$*= 10,701, df=20, p<0,01*). Tukey's post-hoc analysis results are shown in figure 11.

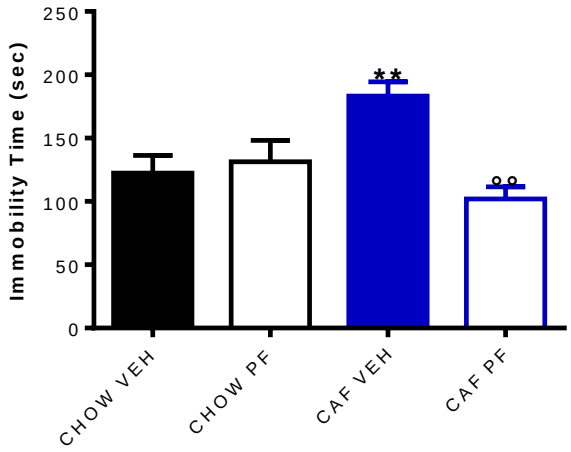

Fig.11: Immobility time in seconds observed during the FST. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; °°p<0,01 vs CAF VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=5; CHOW PF group n=6; CAF VEH group n=5; CAF PF group n=5.

3. IMMUNOFLUORESCENCE STAINING:

Since behavioural tests evidenced mood tone alterations due to the withdrawal from CAFETERIA diet, we decided to focus our attention on the various monoaminergic systems in the brain, in particular in the dopaminergic, noradrenergic and serotoninergic system. Previous studies have evidenced that both anxiety and

depression could be related to monoaminergic transmission impairments in key areas of the brain[129]; moreover, the majority of drugs clinically used up to date with an anxiolytic and anti-depressant effect act on these systems[251,263]. Monoaminergic transmission is also involved in the reward system and gratification[264]; whose impairments exacerbate anxiety-like conditions and addiction[265].

To this purpose, we analysed protein expression by immunofluorescence staining of the enzymes involved in the synthesis of monoamines, such as:

- Tyrosine Hydroxylase (TH) for DA
- Dopamine β- Hydroxylase (DBH) for NA
- Tryptophan Hydroxylase (TPH) for 5HT

In this way, it is possible to discover whether the exposure to CAFETERIA diet or the pharmacological treatment impacted on the expression of the enzymes of synthesis of neurotransmitters.

TH and DBH immunofluorescence were done in double staining with CB1 receptor.

3.1 IMMUNOFLUORESCENCE DOUBLE STAINING TH-CB1:

Immunofluorescence double staining for TH and CB1 receptor was performed in the brain areas of synthesis of DA (VTA and SN) and in areas of projection of DA neurons such as DLS, ACC, DR, PAG.

In VTA, densitometric analysis of TH revealed that PF-3845 treatment per se, in animals not subjected to diet abstinence (CHOW PF group) caused a significant increment of the expression of the enzyme when compared to the control group. Differently, animals treated with PF-3845 and abstinent from the diet (CAF PF) showed a significant decrement of the expression of TH, with respect to CHOW PF animals. Two-way ANOVA revealed no significant effect for the diet ($F_{diet}=3,541, df=12, p=0,087$) and for treatment ($F_{treatment}=1,406, df=12, p=0,261$). Significant interaction between the two factors was detected ($F_{interaction}= 8,660, df=12, p<0,05$). Tukey's post-hoc analysis results are shown in figure 12.

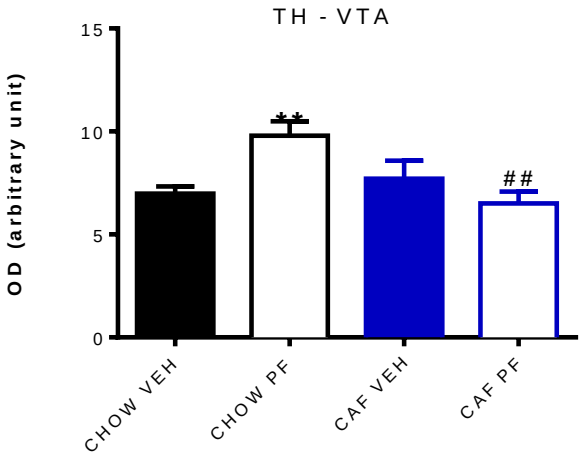

Fig.12: TH densitometric analysis for VTA. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; ##p<0,01 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

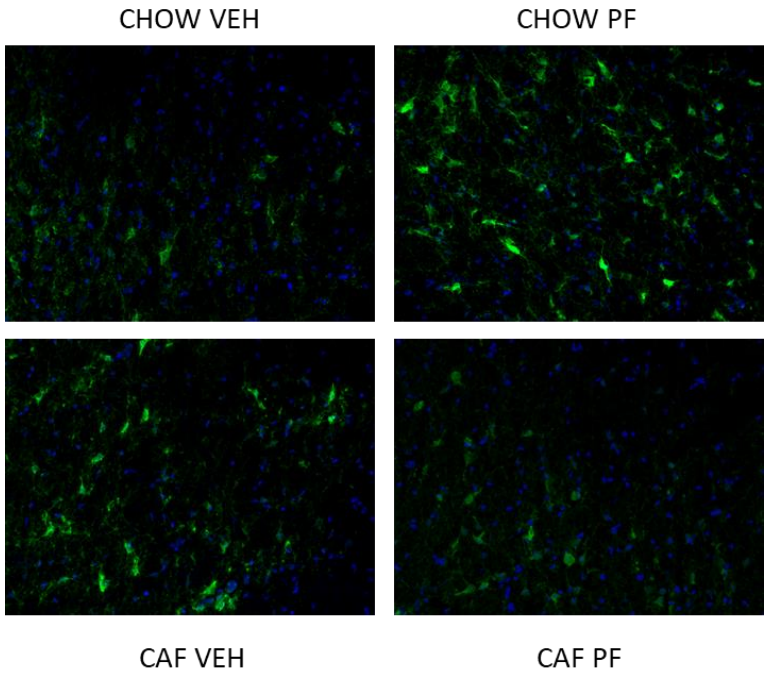

Fig.12A: 20x representative image of TH immunostaining in VTA. TH is coloured in green, DAPI in blue.

In the same region, we quantified also the expression of CB1 receptor: diet abstinence, in CAF VEH group, was significantly able to reduce the expression of CB1 receptor in VTA if compared to CHOW VEH one. Two-way ANOVA revealed significant effect for the diet (F_{diet}*=8,661, df=12, p<0,05*) and not for treatment ($F_{treatment}$*=0,148, df=12, p=0,708*). No significant interaction between the two factors was detected ($F_{interaction}$*= 0,345, df=12, p=0,569*). Tukey's post-hoc analysis results are shown in figure 13.

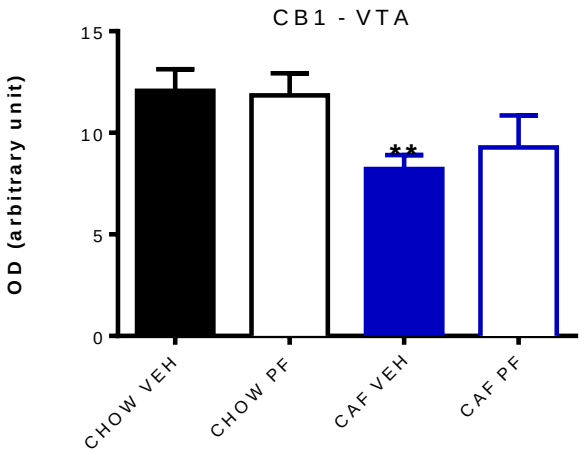

Fig.13: CB1 densitometric analysis for VTA. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

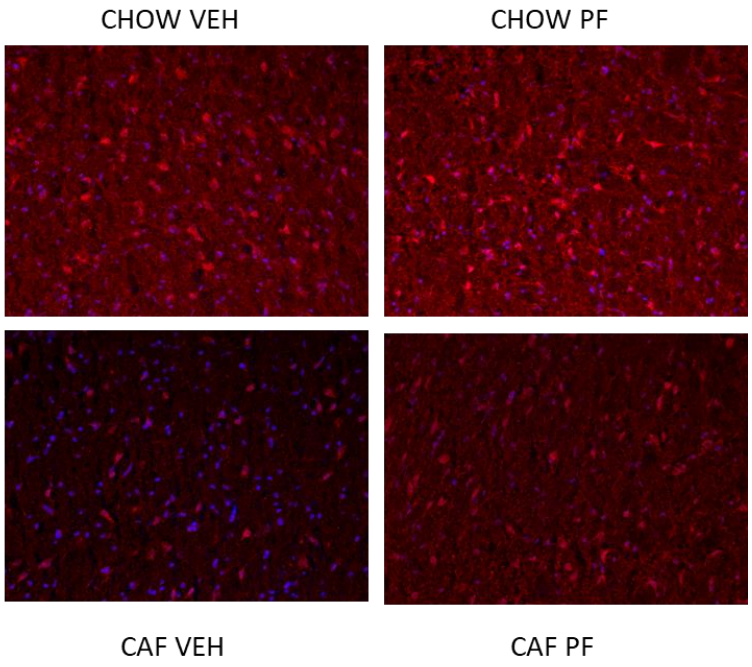

Fig.13A: 20x representative image of CB1 immunostaining in VTA. CB1 is coloured in red, DAPI in blue.

Differently, in SN densitometric analysis of TH revealed a significant decrease of the expression of the protein in CAF PF animals when compared to CHOW PF ones. Two-way ANOVA revealed significant effect for the diet (F_{diet}*=5,486, df=12, p<0,05*) and not for treatment ($F_{treatment}$*=0,132, df=12, p=0,723*). No significant interaction between the two factors was detected ($F_{interaction}$*= 1,591, df=12, p=0,233*). Tukey's post-hoc analysis results are shown in figure 14.

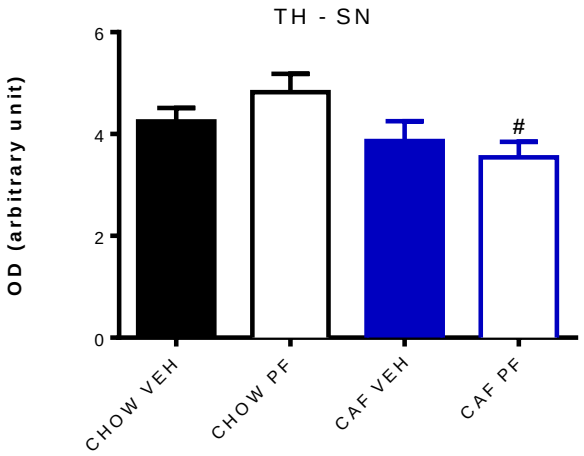

Fig.14: TH densitometric analysis for SN. Results are expressed as MEAN±SEM. $^{\#}p<0,05$ vs CHOW PF (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

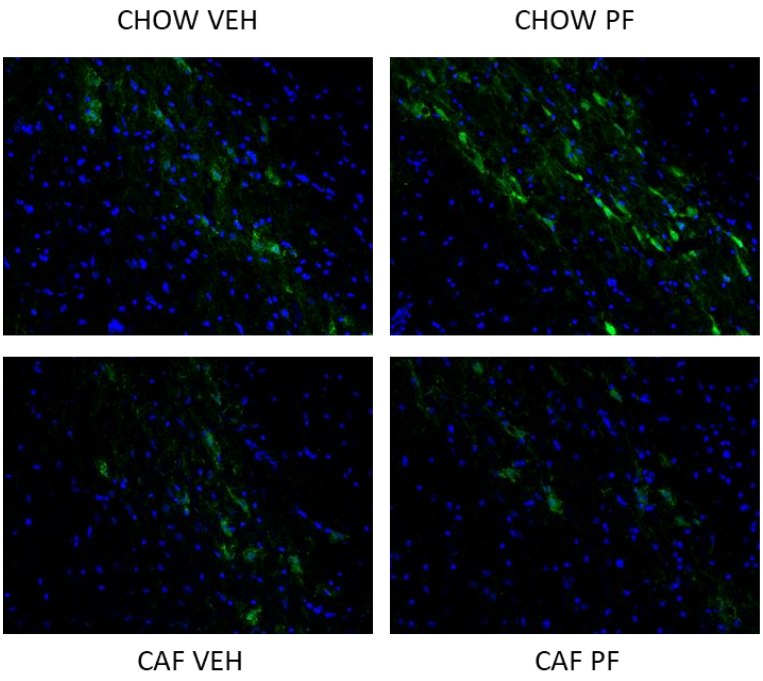

Fig.14A: 20x representative image of TH immunostaining in SN. TH is coloured in green, DAPI in blue.

In the same region, we quantified also the expression of CB1 receptor: diet abstinence, in CAF VEH group was significantly able to increase the expression of CB1 receptor in SN if compared to CHOW VEH one. At the same time, PF-3845 administration significantly increased CB1 expression in CHOW PF animals when compared to CHOW VEH ones. Two-way ANOVA revealed no significant effect for the diet (F_{diet}*=0,214, df=12, p=0,653*) and for treatment ($F_{treatment}$*=0,066, df=12, p=0,802*). Significant

interaction between the two factors was detected ($F_{interaction}$= *6,535, df=12, p<0,05*). Tukey's post-hoc analysis results are shown in figure 15.

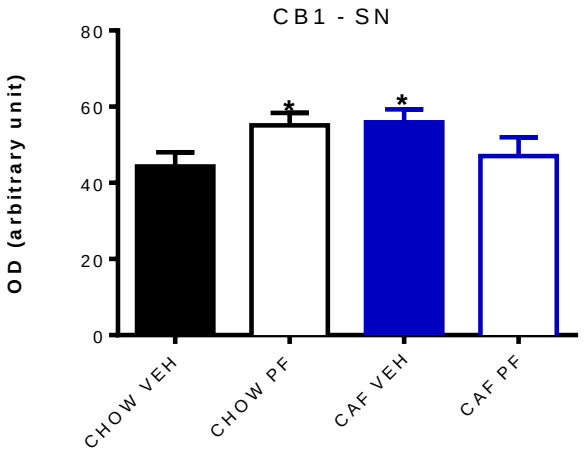

Fig.15: CB1 densitometric analysis for SN. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

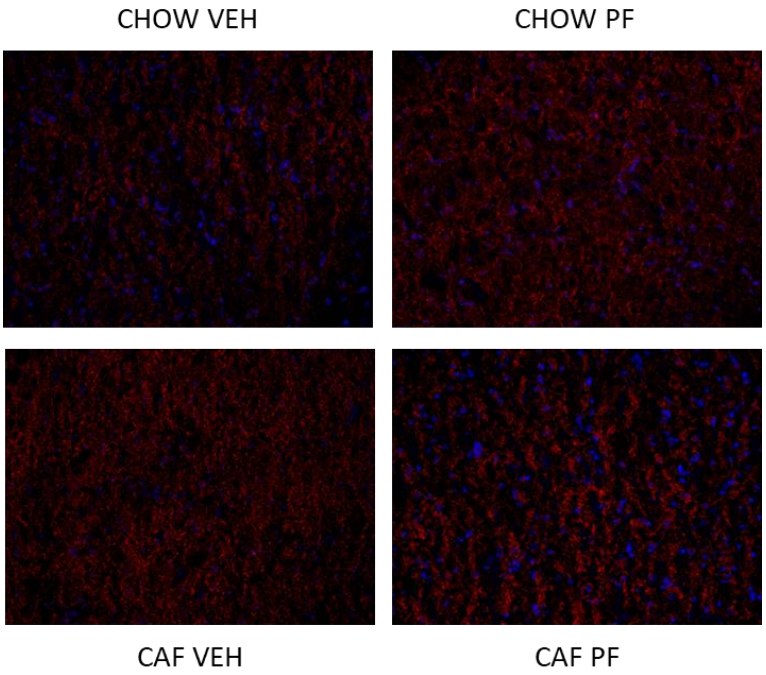

Fig.15A: 20x representative image of CB1 immunostaining in SN. CB1 is coloured in red, DAPI in blue.

In DLS, TH immunostaining presented strong variations between groups: both CHOW PF and CAF VEH groups present a significant decrease of the expression of the protein

when compared to CHOW VEH. Moreover, CAF PF animals present a significant decrease of the expression of TH when compared to CHOW PF ones. Two-way ANOVA revealed significant effect for the diet (F_{diet}*=46,138, df=12, p<0,001*) and for treatment ($F_{treatment}$*=11,425, df=12, p<0,01*). No significant interaction between the two factors was detected ($F_{interaction}$*= 2,244, df=12, p=0,162*). Tukey's post-hoc analysis results are shown in figure 16.

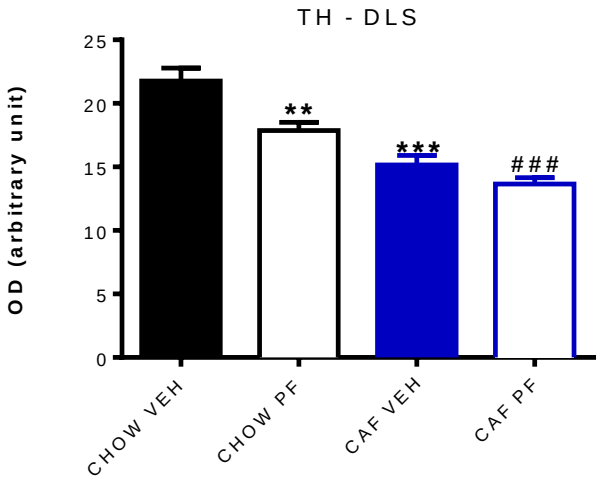

Fig.16: TH densitometric analysis for DLS. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; ***p<0,001 vs CHOW VEH; ###p<0,001 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

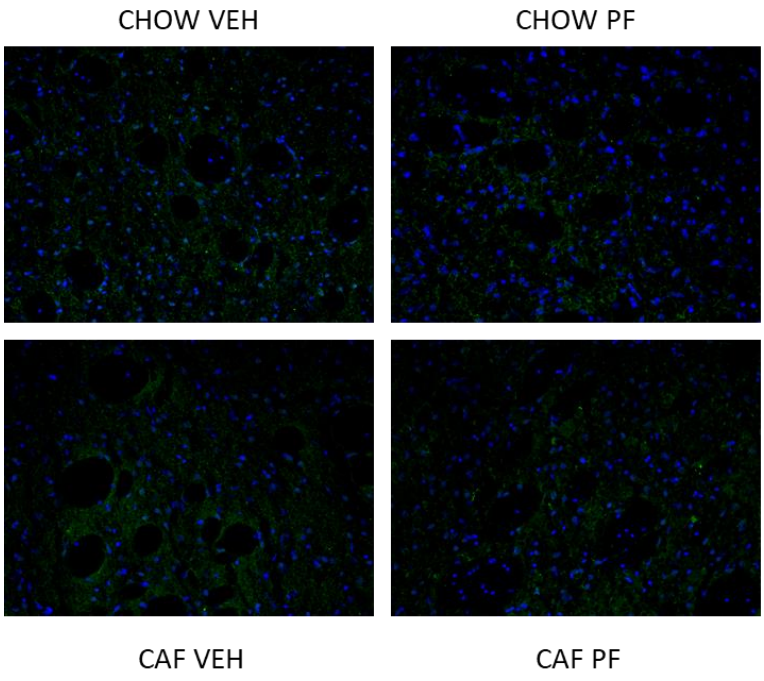

Fig.16A: 20x representative image of TH immunostaining in DLS. TH is coloured in green, DAPI in blue.

In the same region, we also quantified the expression of CB1 receptor: in CAF PF group we noticed a significant decrement of the expression of the receptor compared both to CAF VEH and CHOW PF group. Two-way ANOVA revealed significant effect for the diet (F_{diet}*=17,201, df=12, p<0,01*) and for treatment ($F_{treatment}$*=7,406, df=12, p<0,05*). Significant interaction between the two factors was detected ($F_{interaction}$*= 12,862, df=12, p<0,01*). Tukey's post-hoc analysis results are shown in figure 17.

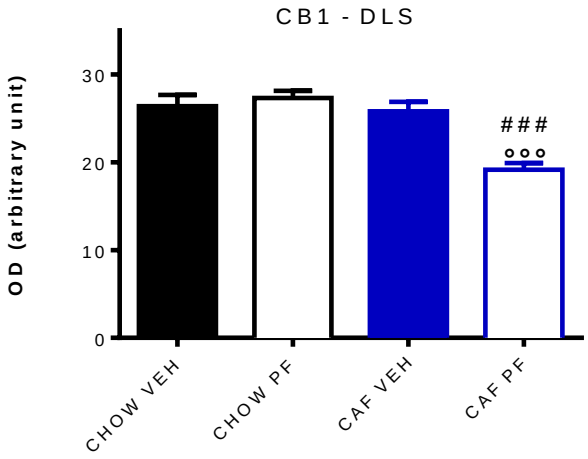

Fig.17: CB1 densitometric analysis for DLS. Results are expressed as MEAN±SEM. °°°p<0,001 vs CAF VEH; ###p<0,001 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

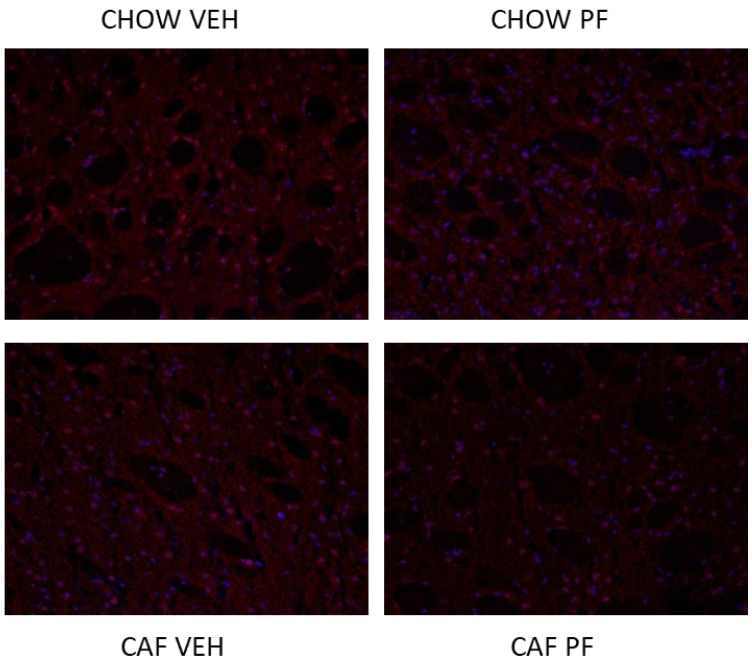

Fig.17A: 20x representative image of CB1 immunostaining in DLS. CB1 is coloured in red, DAPI in blue.

In ACC, TH immunostaining varied between the different groups: both CHOW PF and CAF VEH groups displayed a significant decrease of the expression of TH, when compared to CHOW VEH. Two-way ANOVA revealed significant effect for the diet (F_{diet}*=30,705, df=12, p<0,001*) and for treatment ($F_{treatment}$*=17,235, df= 1/12, p<0,01*). Significant interaction between the two factors was detected ($F_{interaction}$*= 28,888, df= 1/12, p<0,001*). Tukey's post-hoc analysis results are shown in figure 18.

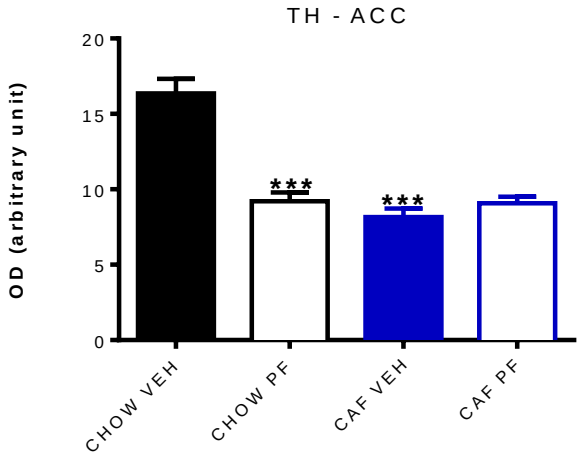

Fig.18: TH densitometric analysis for ACC. Results are expressed as MEAN±SEM. ***p<0,001 vs CHOW VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

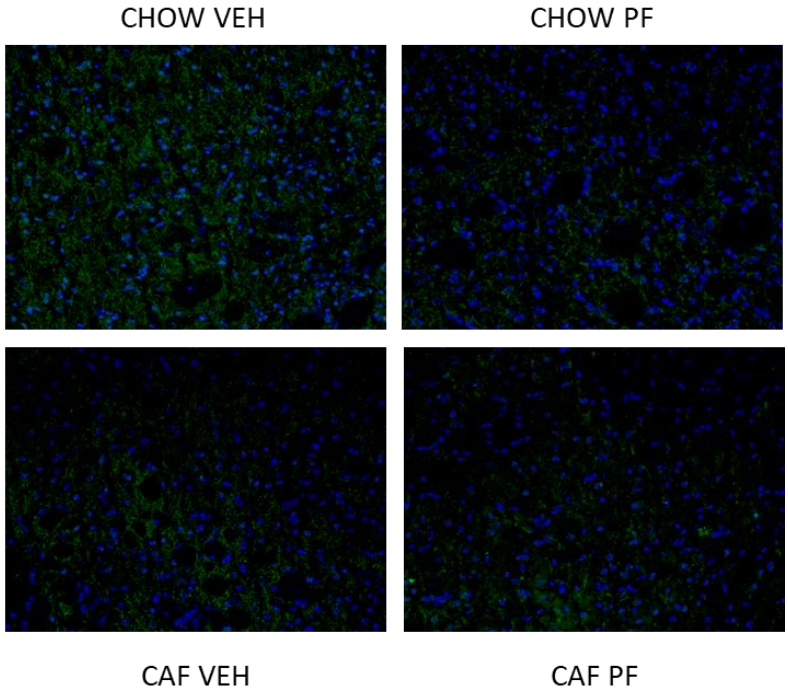

Fig.18A: 20x representative image of TH immunostaining in ACC. TH is coloured in green, DAPI in blue.

In the same region, we quantified also the expression of CB1 receptor: both CHOW PF and CAF VEH animals displayed a significant reduction of the expression of CB1 when compared to CHOW VEH. Moreover, in CAF PF animals CB1 expression is significantly dampened when compared to CHOW PF ones. Two-way ANOVA revealed significant effect for the diet (F_{diet}*=29,113, df=12, p<0,001*) and not for treatment ($F_{treatment}$*=2,939, df=12, p=0,114*). No significant interaction between the two factors was detected ($F_{interaction}$*= 3,883, df=12, p=0,074*). Tukey's post-hoc analysis results are shown in figure 19.

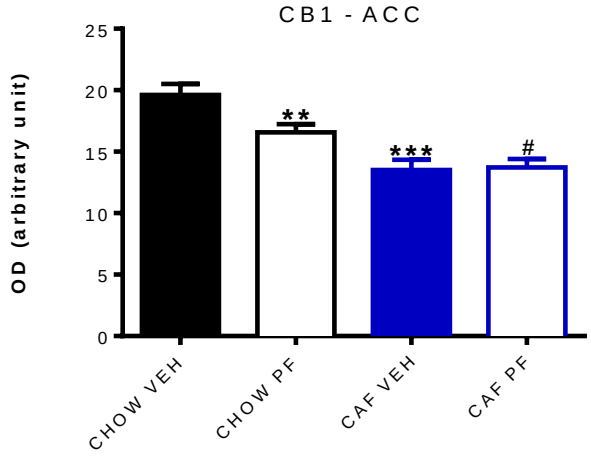

Fig.19: CB1 densitometric analysis for ACC. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; ***p<0,001 vs CHOW VEH; #p<0,05 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

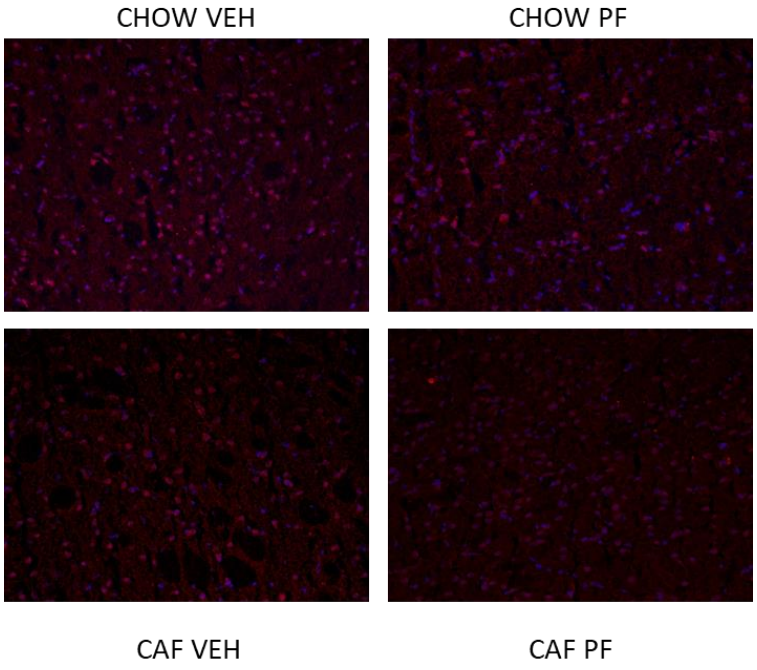

Fig.19A: 20x representative image of CB1 immunostaining in ACC. CB1 is coloured in red, DAPI in blue.

Differently, in DR densitometric analysis of TH revealed a significant increase of the expression of the protein in CAF VEH animals when compared to CHOW VEH ones. Moreover, CAF PF group showed a significant decrease of TH expression when compared to CHOW PF. Two-way ANOVA revealed significant effect for the diet (F_{diet}*=13,678, df=12, p<0,01*) and not for treatment ($F_{treatment}$*=0,787, df=12, p=0,394*). No significant interaction between the two factors was detected ($F_{interaction}$*= 0,638, df=12, p=0,441*). Tukey's post-hoc analysis results are shown in figure 20.

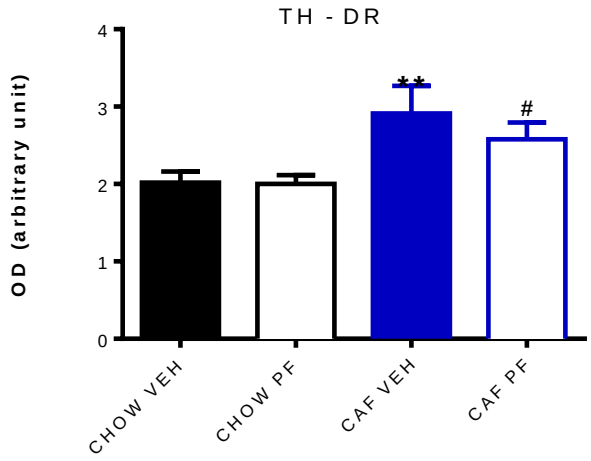

Fig.20: TH densitometric analysis for DR. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; #p<0,05 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

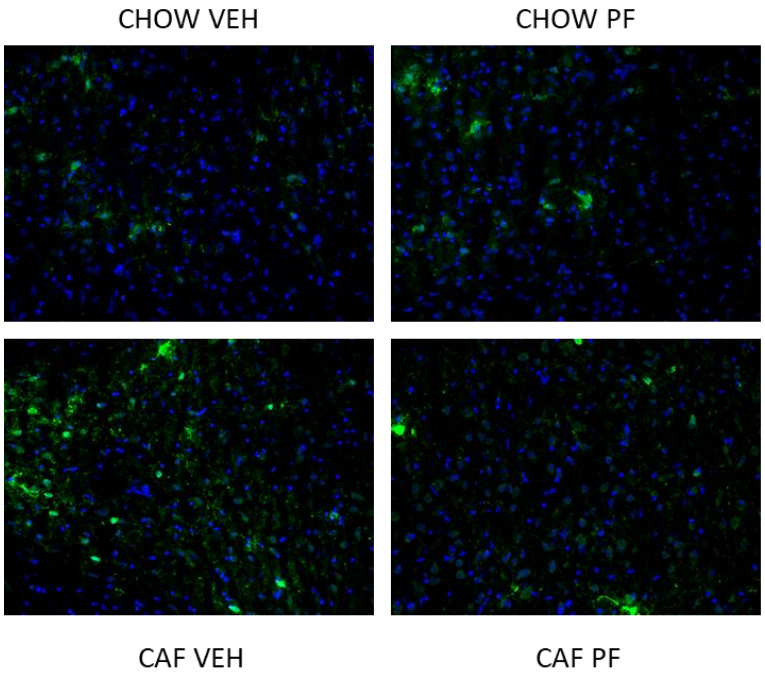

Fig.20A: 20x representative image of TH immunostaining in DR. TH is coloured in green, DAPI in blue.

In the same region, we quantified also the expression of CB1 receptor: both CHOW PF and CAF VEH animals displayed a significant increment of the expression of CB1 when compared to CHOW VEH. Two-way ANOVA revealed no significant effect for the diet (F_{diet}*=4,023, df=12, p=0,070*) and neither for treatment ($F_{treatment}$*=2,111, df=12, p=0,174*). No significant interaction between the two factors was detected ($F_{interaction}$*=2,115, df=12, p=0,174*). Tukey's post-hoc analysis results are shown in figure 21.

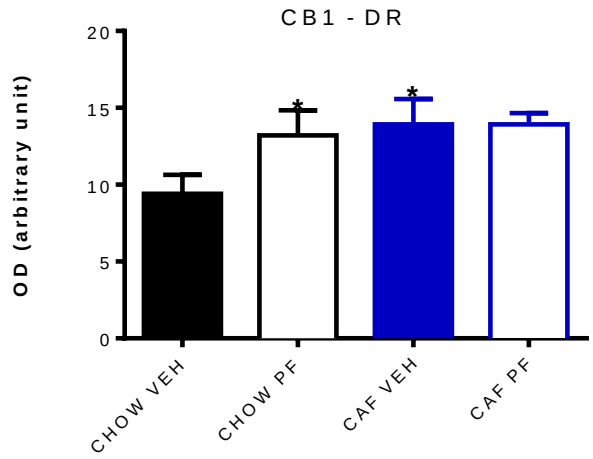

Fig.21: CB1 densitometric analysis for DR. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

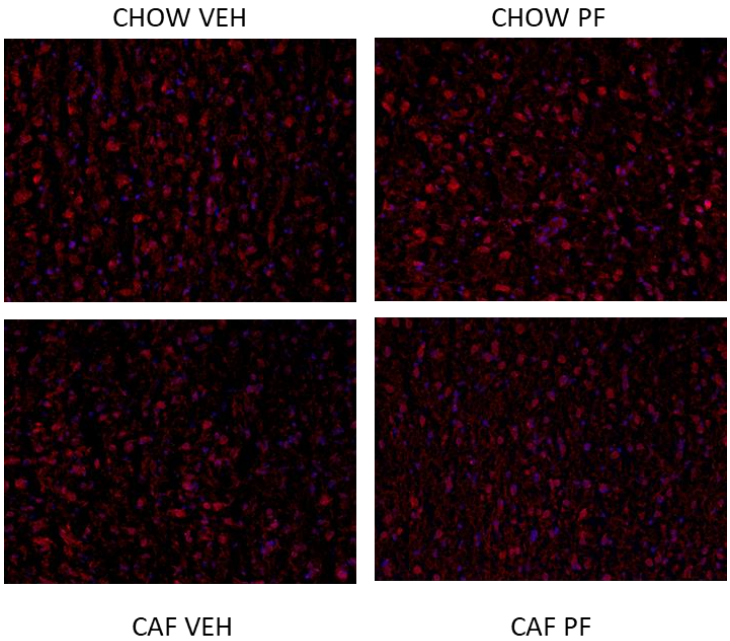

Fig.21A: 20x representative image of CB1 immunostaining in DR. CB1 is coloured in red, DAPI in blue.

In PAG, TH immunostaining varied between the different groups: both CHOW PF and CAF VEH groups displayed a significant increase of the expression of TH, when compared to CHOW VEH. Moreover, CAF PF group showed a significant increase of TH expression when compared to CHOW PF. Two-way ANOVA revealed significant effect for the diet (F_{diet}*=24,044, df=12, p<0,001*) and not for treatment ($F_{treatment}$*=2,762, df=12, p=0,125*). No significant interaction between the two factors was detected ($F_{interaction}$*= 1,752, df= 1/12, p=0,212*). Tukey's post-hoc analysis results are shown in figure 22.

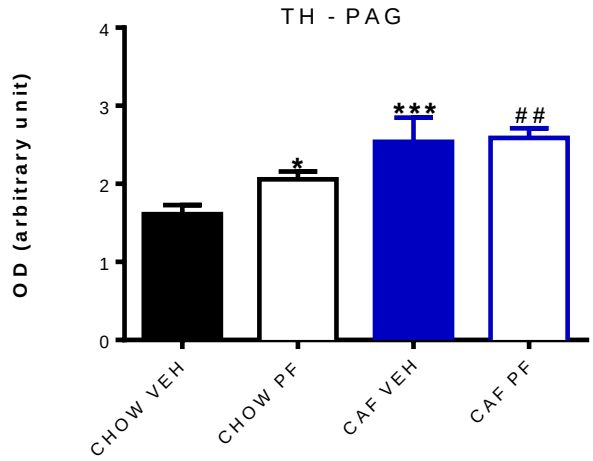

Fig.22: TH densitometric analysis for PAG. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; ***p<0,001 vs CHOW VEH; ##p<0,01 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

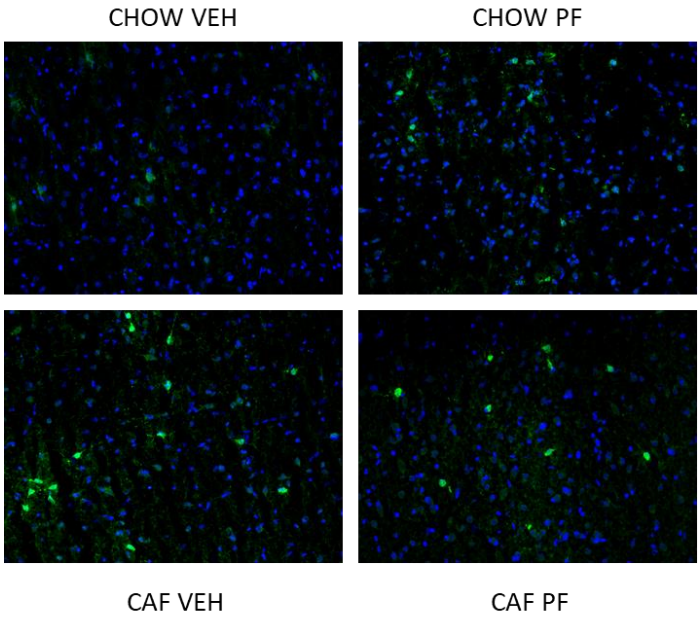

Fig.22A: 20x representative image of TH immunostaining in DR. TH is coloured in green, DAPI in blue.

In the same region, we quantified also the expression of CB1 receptor: CAF VEH animals displayed a significant increment of the expression of CB1 when compared to CHOW VEH. Two-way ANOVA revealed significant effect for the diet (F_{diet}*=5,329, df=12, p<0,05*) and not for treatment ($F_{treatment}$*=0,202, df=12, p=0,662*). No significant interaction between the two factors was detected ($F_{interaction}$*= 0,298, df=12, p=0,596*). Tukey's post-hoc analysis results are shown in figure 23.

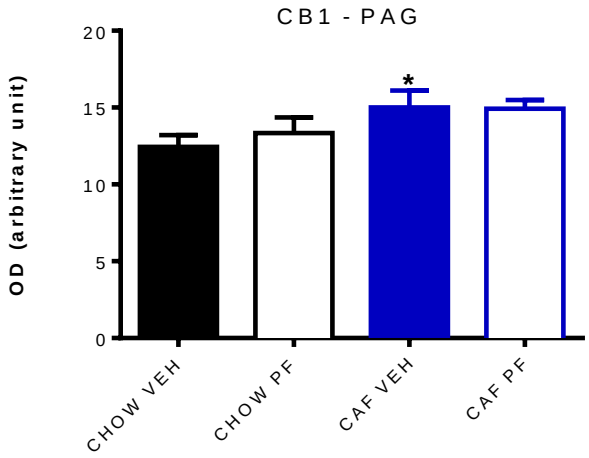

Fig.23: CB1 densitometric analysis for PAG. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

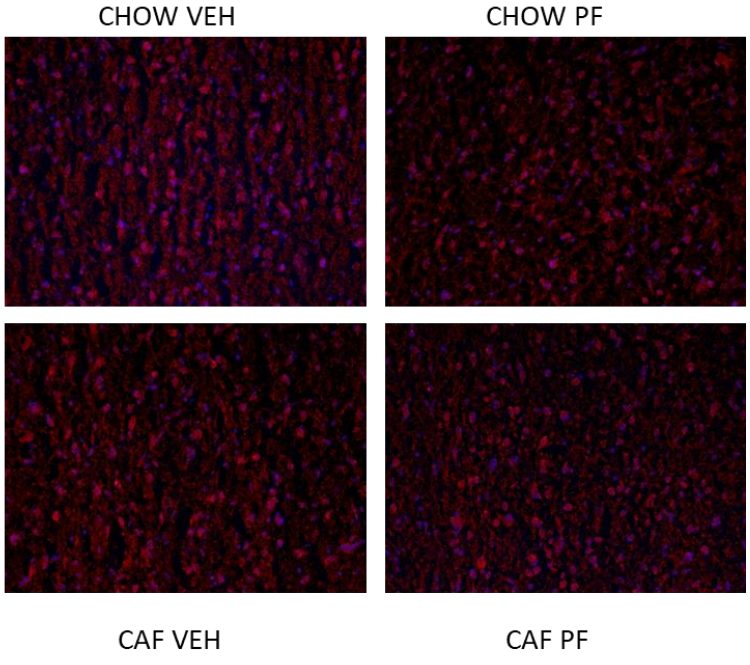

Fig.23A: 20x representative image of CB1 immunostaining in PAG. CB1 is coloured in red, DAPI in blue.

3.2 IMMUNOFLUORESCENCE DOUBLE STAINING DBH-CB1:

Immunofluorescence staining for DBH was only performed in the area of synthesis of NA, that is LC.

DBH immunostaining in LC varied between the different groups: in particular, we noticed a significant increment of DBH expression in CAF VEH and CHOW PF animals when compared to CHOW VEH ones. Besides, CAF rats treated with PF-3845 (CAF PF group) showed a significant increase of DBH when compared to vehicle-treated animals (CAF VEH). Two-way ANOVA revealed no significant effect for the diet (F_{diet}*=3,054, df=12, p=0,108*), but significant effect for treatment ($F_{treatment}$*=30,642, df=12, p<0,001*). No significant interaction between the two factors was detected ($F_{interaction}$*= 1,616, df=12, p=0,230*). Tukey's post-hoc analysis results are shown in figure 24.

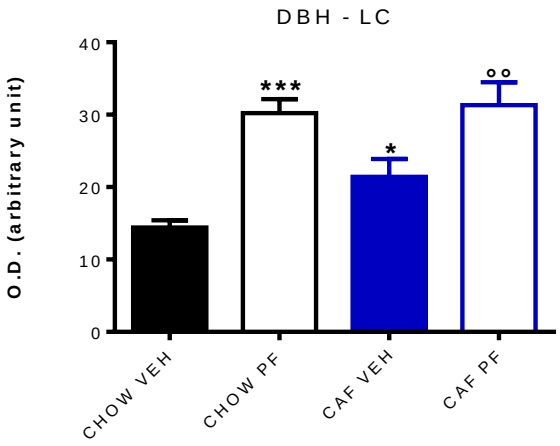

Fig.24: DBH densitometric analysis for LC. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; ***p<0,001 vs CHOW VEH; °°p<0,01 vs CAF VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

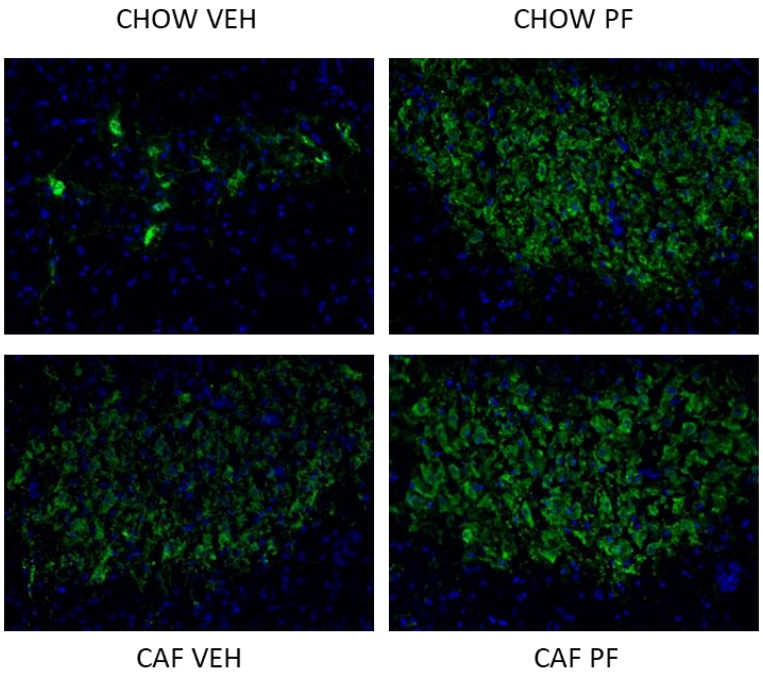

Fig.24A: 20x representative image of DBH immunostaining in LC. DBH is coloured in green, DAPI in blue.

In the same region, we quantified also the expression of CB1 receptor: no significant variations were observed between groups. Two-way ANOVA revealed no significant effect for the diet (F_{diet}*=0,421, df=12, p=0,517*) and neither for treatment ($F_{treatment}$*=4,308, df=12, p=0,062*). No significant interaction between the two factors was detected ($F_{interaction}$*= 0,476, df=12, p=0,505*). Figure 25 shows Tukey's post-hoc analysis results.

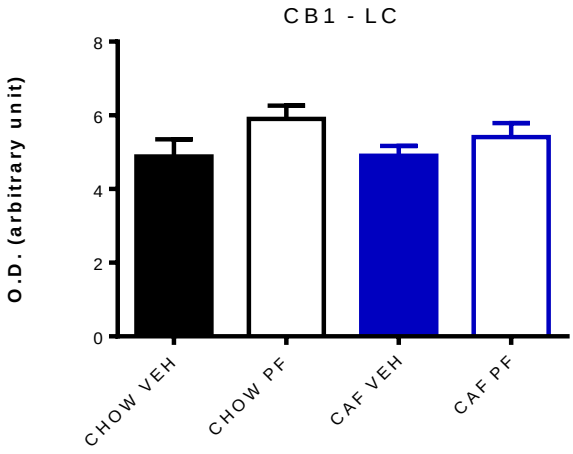

Fig.25: CB1 densitometric analysis for LC. Results are expressed as MEAN±SEM. CHOW VEH group n=3; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

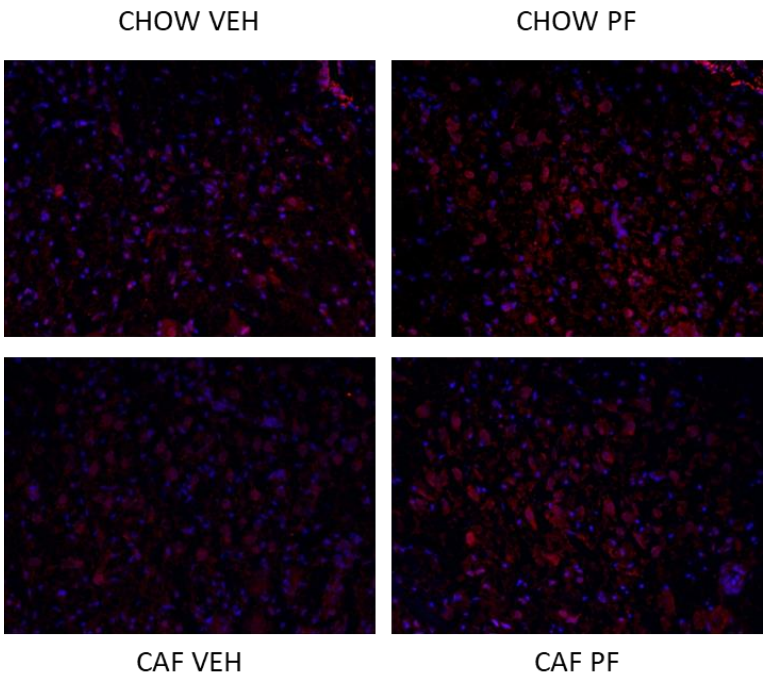

Fig.25A: 20x representative image of CB1 immunostaining in LC. CB1 is coloured in red, DAPI in blue.

3.3 IMMUNOFLUORESCENCE STAINING TPH:

Immunofluorescence staining for TPH was only performed in the area of synthesis of 5HT, that is DR.

TPH immunostaining in DR varied between the different groups: in particular, we noticed a significant increment of DBH expression in CAF VEH and CHOW PF animals when compared to CHOW VEH ones. Two-way ANOVA revealed no significant effect for the diet (F_{diet}*=1,329, df=11, p=0,276*), but significant effect for treatment ($F_{treatment}$*=9,700, df=11, p<0,05*). No significant interaction between the two factors was detected ($F_{interaction}$*= 3,484, df=11, p=0,092*). Tukey's post-hoc analysis results are shown in figure 26.

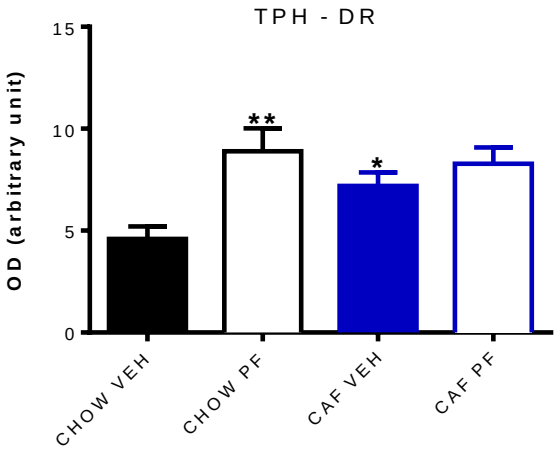

Fig.26: TPH densitometric analysis for DR. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; **p<0,01 vs CHOW VEH (Tukey's post-hoc test for between-groups comparisons). CHOW VEH group n=2; CHOW PF group n=3; CAF VEH group n=3; CAF PF group n=3.

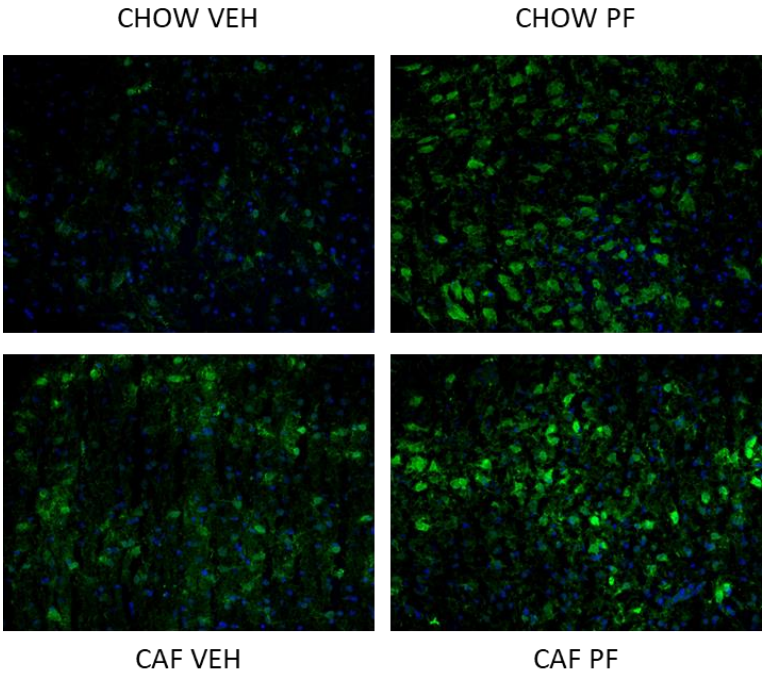

Fig.26A: 20x representative image of TPH immunostaining in LC. TPH is coloured in green, DAPI in blue.

4. HPLC ANALYSIS:

Based on the results obtained by behavioural study and on the results regarding the expressions of the enzymes involved in the synthesis of monoamines, we decided to analyse monoaminergic (DA, NA, 5HT) tone in different brain regions, and in particular:

- areas of synthesis of neurotransmitters (VTA and SN for DA, DR for 5HT, LC for NA);
- areas involved in the reward system (DLS, ACC, AMY, PFC and mPFC);
- areas involved in the control of pain (PAG);
- areas involved in learning and memory (dHIPPO and vHIPPO);
- areas involved in the control of food intake (HYPO).

For each area we measured tissue concentration of NA, DA, 5-HT and the concentration of DOPAC and HVA (DA main metabolites) and 5HIAA (5HT main metabolite); the turnover ratio was calculated for each neurotransmitter as the ratio between the concentration of the metabolites and the concentration of the neurotransmitter.

4.1 DOPAMINERGIC TRANSMISSION:

For each area cited above, tissue level of dopamine (DA) and its main metabolites 3,4-diidroxyphenilacetic acid (DOPAC) and homovanillic acid (HVA) were detected. Moreover, for each area, we calculated also the turnover of DA, as the ratio DOPAC+HVA/DA. We observed consistent and significant variations of the concentration of DA in different brain areas: in particular abstinence from the diet caused a significant decrease of DA in DR in CAF VEH animals when compared to CHOW VEH rats. In CHOW PF animals, the administration of PF-3845 was able to significantly decrease the concentration of DA in vHIPPO when compared to CHOW VEH group. A significant increase of DA was observed in DLS in CAF PF animals when compared to CHOW PF rats.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 1, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 27.

Two-way ANOVA for the quantity of DA in the different areas

	F diet		F treatment		F interaction		df
PFC	0,008	(p=0,929)	0,089	(p=0,769)	1,143	(p=0,300)	21
mPFC	0,006	(p=0,940)	0,000	(p=0,991)	2,515	(p=0,131)	21
ACC	0,157	(p=0,697)	0,330	(p=0,573)	0,275	(p=0,607)	20
DLS	2,143	(p=0,140)	0,052	(p=0,822)	2,644	(p=0,124)	20
AMY	0,102	(p=0,753)	0,516	(p=0,482)	4,136	(p=0,058)	21
HIPO	0,451	(p=0,511)	0,341	(p=0,567)	3,093	(p=0,097)	21
VPL	0,252	(p=0,622)	2,265	(p=0,152)	0,478	(p=0,499)	20
dHYPPO	1,617	(p=0,221)	0,036	(p=0,853)	0,107	(p=0,747)	21
vHYPPO	1,876	(p=0,189)	2,415	(p=0,139)	2,063	(p=0,169)	21
PAG	0,133	(p=0,721)	1,100	(p=0,310)	0,023	(p=0,881)	20
DR	***4,665***	***(p<0,05)***	0,314	(p=0,583)	1,469	(p=0,242)	21
VTA	0,016	(p=0,901)	0,443	(p=0,515)	***4,898***	***(p<0,05)***	21
SN	1,297	(p=0,271)	0,618	(p=0,443)	1,039	(p=0,322)	21
LC	0,003	(p=0,957)	0,337	(p=0,570)	0,225	(p=0,641)	20
LPB	1,129	(p=0,304)	0,436	(p=0,518)	0,496	(p=0,492)	20

Table 1: Two-way ANOVA analysis results for the concentration of DA in different brain areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). The analysis evidenced diet as a significant factor in DR. A significant interaction between the two factors was detected in VTA. All significant values are written in italics in the table.

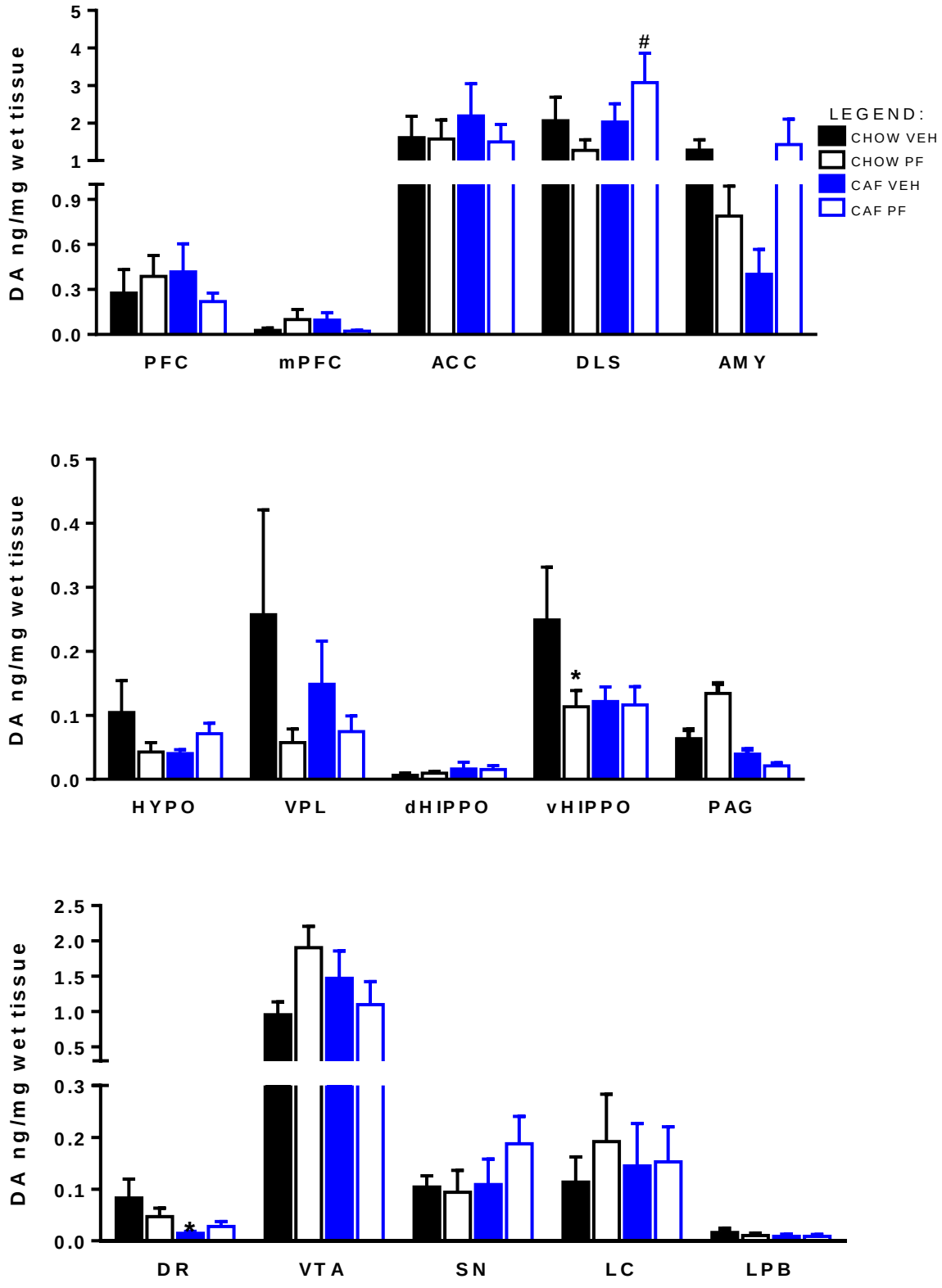

Fig.27: DA concentration values for each area expressed in ng/mg of wet tissue. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; #p<0,05 vs CHOW PF (Tukey's post-hoc test for between groups comparisons).

The HPLC analysis of DOPAC, one of the most important DA metabolites, revealed only a significant variation in HYPO: CAF PF animals display in this region a significant decrease of the concentration of DOPAC, when compared to CHOW PF ones.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 2, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 28.

Two-way ANOVA for the quantity of DOPAC in the different areas

	F diet		F treatment		F interaction		df
PFC	0,063	(p=0,805)	0,931	(p=0,348)	1,873	(p=0,189)	21
mPFC	0,408	(p=0,531)	0,878	(p=0,362)	3,046	(p=0,099)	21
ACC	0,015	(p=0,904)	1,444	(p=0,247)	0,205	(p=0,657)	20
DLS	0,020	(p=0,889)	0,029	(p=0,866)	0,760	(p=0,396)	20
AMY	2,079	(p=0,168)	0,223	(p=0,643)	0,004	(p=0,948)	21
HIPO	***7,900***	***(p<0,05)***	0,047	(p=0,831)	0,962	(p=0,340)	21
VPL	0,117	(p=0,737)	2,864	(p=0,110)	0,135	(p=0,718)	20
dHYPPO	0,260	(p=0,616)	0,003	(p=0,954)	0,580	(p=0,457)	21
vHYPPO	3,709	(p=0,071)	1,378	(p=0,257)	0,107	(p=0,748)	21
PAG	0,000	(p=0,987)	0,862	(p=0,367)	0,169	(p=0,686)	20
DR	1,511	(p=0,236)	1,956	(p=0,180)	0,858	(p=0,367)	21
VTA	0,168	(p=0,687)	0,005	(p=0,945)	1,213	(p=0,286)	21
SN	0,076	(p=0,786)	0,029	(p=0,867)	0,078	(p=0,784)	21
LC	0,845	(p=0,372)	0,015	(p=0,905)	0,040	(p=0,845)	20
LPB	1,982	(p=0,178)	0,121	(p=0,732)	0,445	(p=0,514)	20

Table 2: Two-way ANOVA analysis results for the concentration of DOPAC in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). The analysis evidenced diet as a significant factor in HYPO. Treatment was a not significant factor in all the areas. No significant interaction between the two factors was detected in all the areas. All significant values are written in italics in the table.

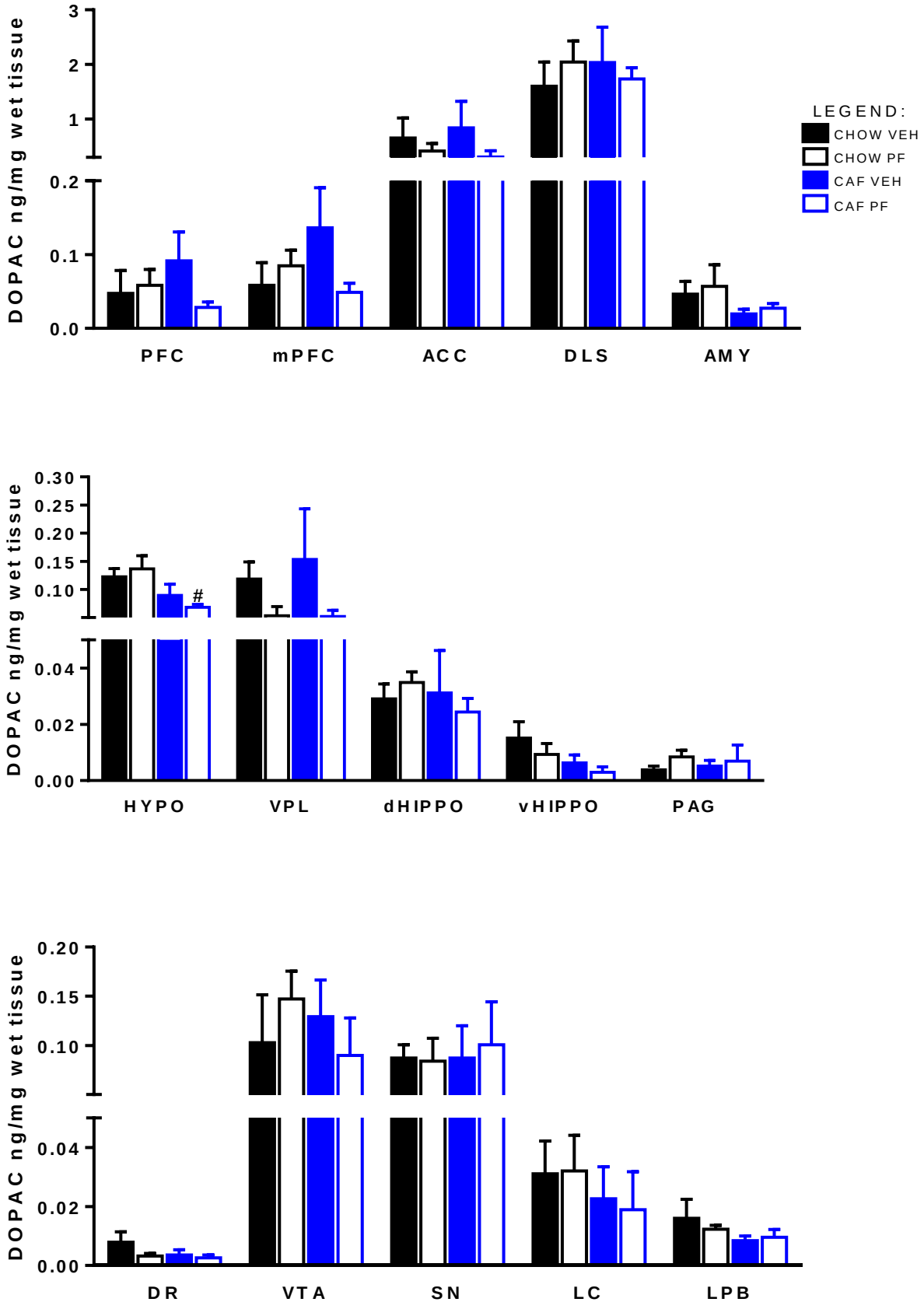

Fig.28: DOPAC concentration values for each area expressed in ng/mg of wet tissue. Results are expressed as MEAN±SEM.; $^{\#}p<0,05$ vs CHOW PF (Tukey's post-hoc test for between-groups comparisons).

HPLC analysis of DA metabolite HVA revealed a significant increment in the concentration of HVA in CAF PF animals in AMY, when compared to CAF VEH treated rats. At the same time, the same CAF PF group showed a significant increase in HVA concentrations in LC when compared to CHOW PF one. These variations of the metabolite HVA once again were linked to an increase of the concentration of DA in the same areas, suggesting that more DA was being produced and metabolized at the same time. Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 3, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 29.

Two-way ANOVA for the quantity of HVA in the different areas

	F diet		F treatment		F interaction		df
PFC	0,344	(p=0,565)	0,166	(p=0,689)	1,209	(p=0,287)	21
mPFC	0,634	(p=0,437)	0,009	(p=0,926)	1,194	(p=0,290)	21
ACC	0,285	(p=0,601)	1,291	(p=0,273)	0,030	(p=0,864)	20
DLS	0,000	(p=0,994)	0,832	(p=0,375)	0,000	(p=0,991)	20
AMY	0,364	(p=0,554)	3,490	(p=0,079)	2,392	(p=0,140)	21
HIPO	2,836	(p=0,110)	1,692	(p=0,211)	0,073	(p=0,790)	21
VPL	0,269	(p=0,611)	1,766	(p=0,202)	0,007	(p=0,936)	20
dHYPPO	0,118	(p=0,735)	0,801	(p=0,383)	0,010	(p=0,923)	21
vHYPPO	3,225	(p=0,090)	1,254	(p=0,278)	0,938	(p=0,346)	21
PAG	0,582	(p=0,457)	1,227	(p=0,284)	0,349	(p=0,563)	20
DR	0,958	(p=0,341)	0,510	(p=0,485)	0,047	(p=0,831)	21
VTA	0,001	(p=0,970)	0,490	(p=0,494)	2,983	(p=0,102)	21
SN	1,011	(p=0,329)	2,001	(p=0,175)	0,560	(p=0,464)	21
LC	**11,513**	***(p<0,01)***	2,407	(p=0,140)	1,989	(p=0,178)	20
LPB	0,210	(p=0,653)	0,085	(p=0,775)	0,800	(p=0,384)	20

Table 3: Two-way ANOVA analysis results for the concentration of HVA in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). The analysis evidenced diet as a significant factor in LC. No significant interaction between the two factors was detected in all the areas.

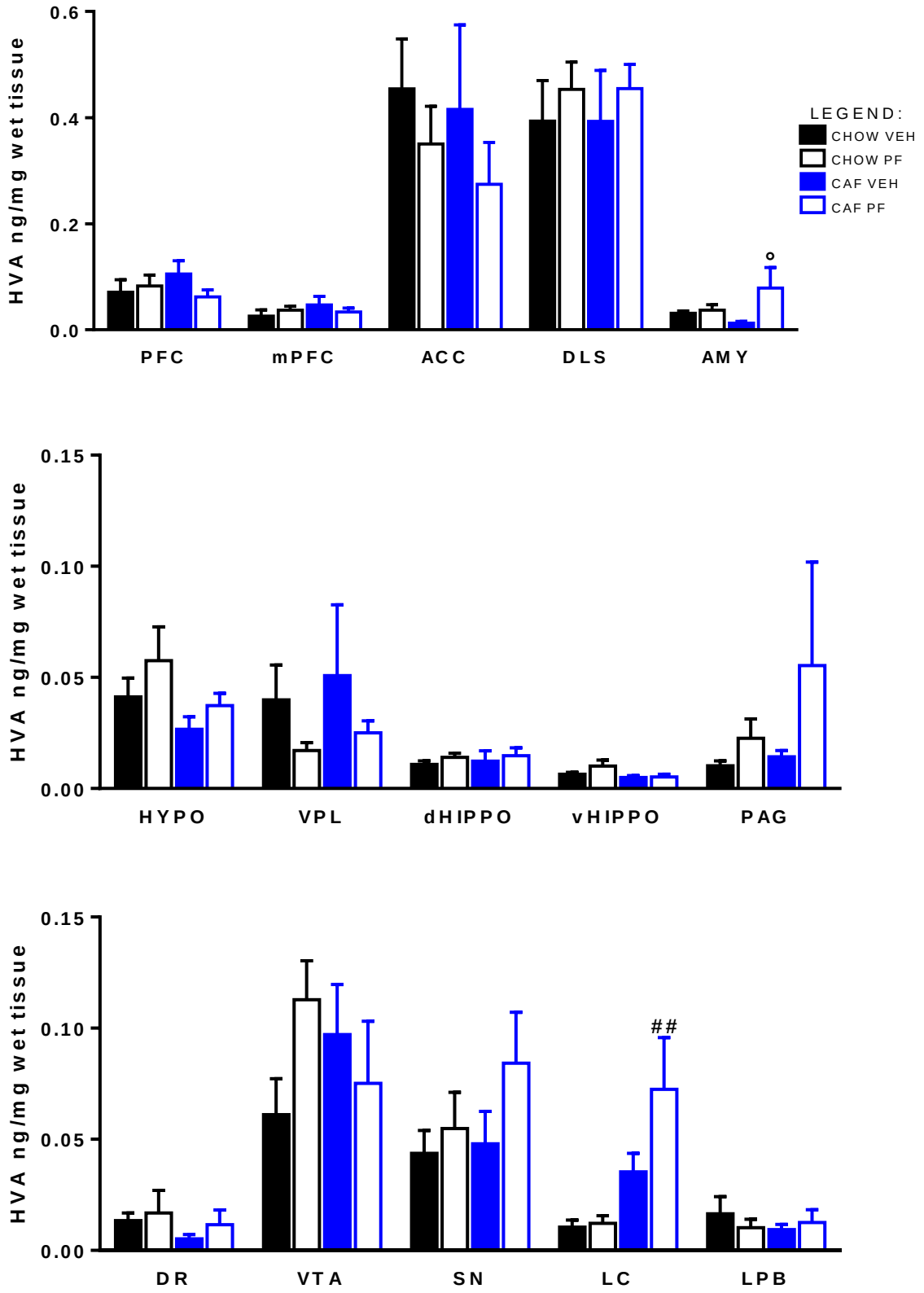

Fig.29: HVA concentration values for each area expressed in ng/mg of wet tissue. Results are expressed as MEAN±SEM. $^{\#\#}p<0,01$ vs CHOW PF; °$p<0,05$ vs CAF VEH (Tukey's post-hoc test for between-groups comparisons).

DA turnover was analysed as the ratio between the concentration of DOPAC+HVA and DA. No significant variation of DA turnover was observed in consequence of diet withdrawal. On the other hand, CAF PF rats showed a significant decrement of DA turnover in HYPO and vHIPPO when compared to CHOW PF rats.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 4, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 30.

Two-way ANOVA for DA turnover in the different areas

	F diet		F treatment		F interaction		df
PFC	0,637	(p=0,436)	0,632	(p=0,438)	0,084	(p=0,775)	21
mPFC	0,326	(p=0,576)	0,855	(p=0,368)	1,246	(p=0,280)	21
ACC	1,324	(p=0,267)	0,047	(p=0,831)	0,608	(p=0,447)	20
DLS	1,680	(p=0,213)	1,271	(p=0,276)	1,929	(p=0,184)	20
AMY	0,062	(p=0,807)	1,165	(p=0,296)	0,833	(p=0,374)	21
HIPO	2,293	(p=0,148)	1,421	(p=0,250)	2,275	(p=0,150)	21
VPL	0,590	(p=0,454)	0,833	(p=0,375)	0,484	(p=0,496)	20
dHYPPO	2,716	(p=0,118)	1,763	(p=0,202)	1,706	(p=0,209)	21
vHYPPO	***4,644***	***(p<0,05)***	0,139	(p=0,714)	1,385	(p=0,255)	21
PAG	1,889	(p=0,188)	0,032	(p=0,861)	0,020	(p=0,891)	20
DR	2,295	(p=0,148)	0,321	(p=0,578)	0,183	(p=0,674)	21
VTA	0,013	(p=0,910)	0,376	(p=0,548)	0,463	(p=0,505)	21
SN	0,861	(p=0,367)	0,774	(p=0,391)	1,104	(p=0,308)	21
LC	2,344	(p=0,145)	0,282	(p=0,603)	0,844	(p=0,372)	20
LPB	0,526	(p=0,479)	0,653	(p=0,431)	0,770	(p=0,393)	20

Table 4: Two-way ANOVA analysis results for DA turnover in the various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Diet was a significant factor in vHIPPO. No significant interaction between the two factors was detected. All significant values are written in italics in the table.

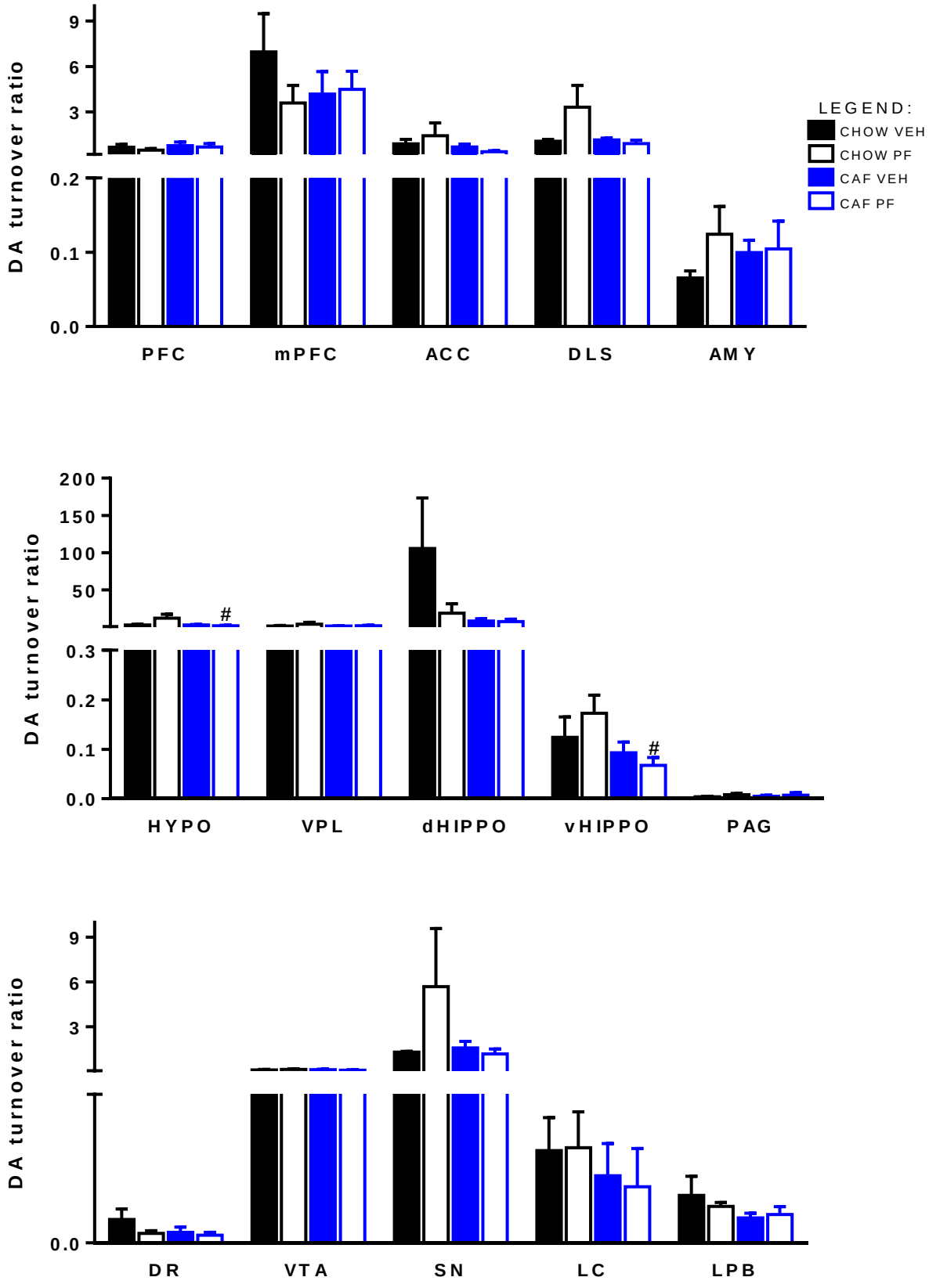

Fig.30: DA turnover ratio values for each area. Results are expressed as MEAN±SEM. $^{\#}p<0,05$ vs CHOW PF (Tukey's post-hoc test for between-groups comparisons).

4.2 NORADRENERGIC TRANSMISSION:

For each of the area cited above, tissue level of noradrenaline (NA) was detected.

The analysis of the quantities of NA in the various areas revealed that this system was less affected than the dopaminergic one by CAFETERIA diet withdrawal: in CAF VEH animals, abstinent from the diet, we generally observed a trend in reduction for the concentration of NA in most of the areas, but a significant decrease of NA concentration in CAF VEH rats was detected in VPL when compared to CHOW VEH ones. The treatment with PF-3845 was significantly able to increase the concentration of NA in AMY in animals abstinent from the diet (CAF PF), compared to animals which did not receive the pharmacological treatment (CAF VEH). PF-3845 administration per se, in CHOW PF group caused a significant decrease of NA concentration in VPL, when compared to CHOW VEH rats.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 5, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 31.

Two-way ANOVA for the quantity of NA in the different areas

	F diet		F treatment		F interaction		df
PFC	0,198	(p=0,662)	0,549	(p=0,469)	0,044	(p=0,836)	21
mPFC	0,003	(p=0,956)	0,061	(p=0,807)	0,084	(p=0,775)	21
ACC	1,297	(p=0,271)	0,402	(p=0,535)	1,463	(p=0,244)	20
DLS	4,164	(p=0,058)	0,005	(p=0,945)	0,262	(p=0,616)	20
AMY	0,021	(p=0,886)	1,123	(p=0,304)	***6,265***	***(p<0,05)***	21
HYPO	0,039	(p=0,845)	0,062	(p=0,806)	1,660	(p=0,215)	21
VPL	3,333	(p=0,087)	1,226	(p=0,285)	***9,830***	***(p<0,05)***	20
dHIPPO	0,228	(p=0,639)	0,020	(p=0,890)	0,095	(p=0,761)	21
vHIPPO	0,865	(p=0,365)	2,402	(p=0,140)	0,450	(p=0,511)	21
PAG	0,003	(p=0,957)	3,927	(p=0,065)	0,727	(p=0,406)	20
DR	2,378	(p=0,141)	0,019	(p=0,891)	0,765	(p=0,394)	21
VTA	3,655	(p=0,073)	0,654	(p=0,430)	0,022	(p=0,884)	21
SN	0,665	(p=0,426)	0,275	(p=0,607)	2,542	(p=0,129)	21
LC	0,407	(p=0,532)	0,007	(p=0,936)	0,395	(p=0,539)	20
LPB	0,103	(p=0,753)	0,000	(p=0,992)	0,177	(p=0,679)	20

Table 5: Two-way ANOVA analysis results for NA in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Significant interaction between the two factors was detected in VPL and AMY. All significant values are written in italics in the table.

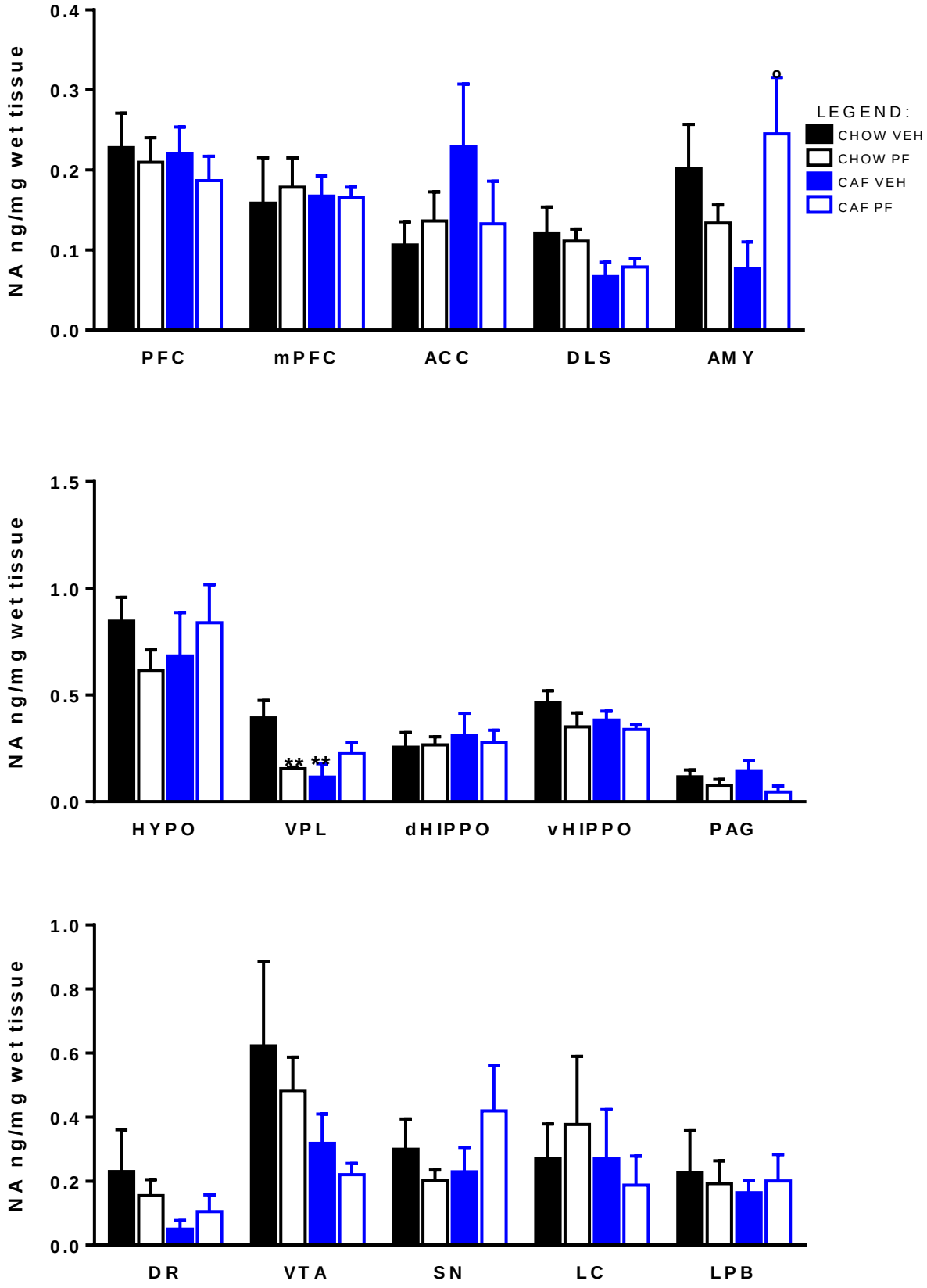

Fig.31: NA concentration values for each area expressed in ng/mg of wet tissue. Results are expressed as MEAN±SEM. **p<0,01 vs CHOW VEH; °p<0,05 vs CAF VEH (Tukey's post-hoc test for between-groups comparisons).

4.3 SEROTONINERGIC TRANSMISSION:

For each of the areas cited above, tissue level of serotonin (5HT) and its main metabolite 5-hydroxyindolacetic acid (5HIAA) were detected. Moreover, for each area, we calculated also the turnover of 5HT, as the ratio 5HIAA/5HT. Referring to 5HT, the abstinence from the diet causes in CAF VEH animals a general trend in decrease for the concentration of the neurotransmitter in the majority of the areas compared to CHOW VEH ones; except for dHIPPO, VTA, LC, LPB where we observed a trend in the increase of the concentration of serotonin if compared to the control group.

Pharmacologic treatment per se, in chow-fed animals (CHOW PF group), was significantly able to decrease the concentration of 5HT in VPL and vHIPPO when compared to CHOW VEH ones; whereas its administration in animals which experienced the withdrawal (CAF PF group) was significantly able to raise the amount of the neurotransmitter in SN when compared both to CAF VEH and CHOW PF group. Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 6, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 32.

Two way ANOVA for the quantity of 5HT in the different areas

	F diet	F treatment	F interaction	df
PFC	3,319 (p=0,086)	0,645 (p=0,433)	1,060 (p=0,318)	21
mPFC	0,384 (p=0,543)	0,000 (p=0,990)	0,022 (p=0,883)	21
ACC	0,089 (p=0,769)	0,222 (p=0,644)	0,489 (p=0,495)	20
DLS	0,200 (p=0,661)	0,136 (p=0,717)	2,888 (p=0,109)	20
AMY	0,396 (p=0,537)	0,029 (p=0,867)	2,153 (p=0,161)	21
HYPO	0,101 (p=0,755)	0,084 (p=0,776)	3,379 (p=0,084)	21
VPL	0,342 (p=0,567)	0,666 (p=0,427)	***5,338 (p<0,05)***	20
dHIPPO	1,238 (p=0,281)	0,420 (p=0,526)	0,463 (p=0,506)	21
vHIPPO	1,524 (p=0,234)	1,867 (p=0,190)	2,454 (p=0,136)	21
PAG	0,580 (p=0,457)	0,809 (p=0,382)	1,123 (p=0,305)	20
DR	3,717 (p=0,071)	0,179 (p=0,677)	0,157 (p=0,697)	21
VTA	0,017 (p=0,897)	0,529 (p=0,477)	1,365 (p=0,259)	21
SN	0,890 (p=0,359)	3,019 (p=0,100)	***5,856 (p<0,05)***	21
LC	0,396 (p=0,538)	0,026 (p=0,873)	0,249 (p=0,625)	20
LPB	0,404 (p=0,534)	0,507 (p=0,487)	0,291 (p=0,597)	20

Table 6: Two-way ANOVA analysis results for 5HT in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Significant interaction between the two factors was detected in VPL and SN. All significant values are written in italics in the table.

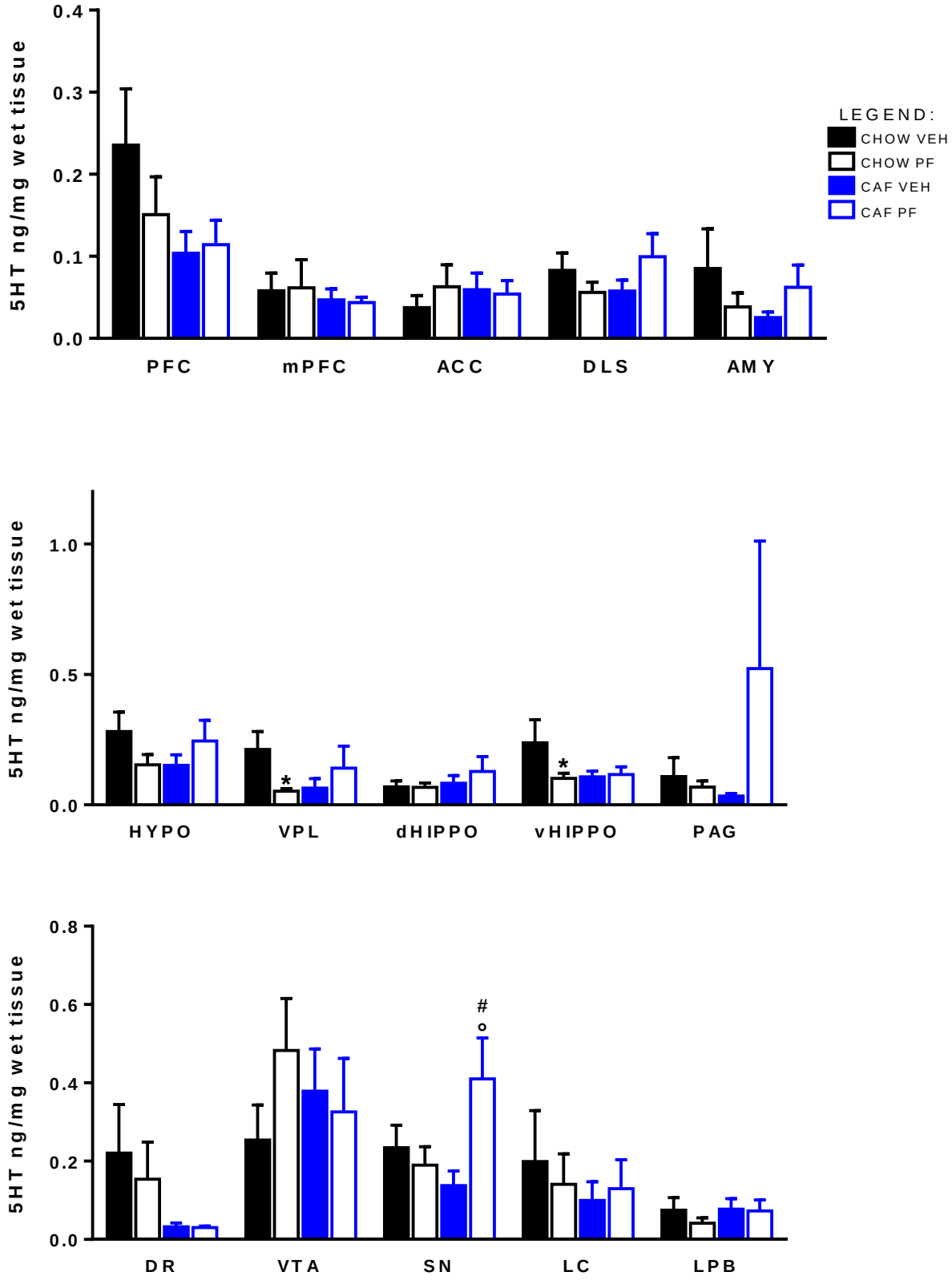

Fig.32: 5HT concentration values for each area expressed in ng/mg of wet tissue. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; °p<0,05 vs CAF VEH; #p<0,05 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons).

Also the concentration of 5HT main metabolite, the 5HIAA, was influenced: abstinence provoked the significant decrease of the concentration of 5HIAA in VPL in CAF VEH rats when compared to CHOW VEH ones; since in these areas less serotonin was produced and consequently metabolized. Differently, in CHOW PF rats PF-3845 administration was able to significantly increase the concentration of the metabolite in PFC and vHIPPO; significantly dampened it in VPL when compared to CHOW VEH. On the other hand, CAF PF rats showed in VTA and PFC a significant decrease of the concentration of 5HIAA when compared to CHOW PF ones.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 7, whereas the results from the post hoc analyses (Tukey's test) are reported in figure 33.

Two-way ANOVA for the quantity of 5HIAA in the different areas

	F diet		F treatment		F interaction		df
PFC	0,204	(p=0,658)	0,308	(p=0,586)	**6,459**	***(p<0,05)***	21
mPFC	0,509	(p=0,485)	2,890	(p=0,107)	0,001	(p=0,976)	21
ACC	1,362	(p=0,260)	0,950	(p=0,344)	0,916	(p=0,353)	20
DLS	2,046	(p=0,172)	0,678	(p=0,422)	0,008	(p=0,928)	20
AMY	0,088	(p=0,770)	0,190	(p=0,669)	4,006	(p=0,062)	21
HYPO	1,950	(p=0,181)	0,002	(p=0,966)	0,464	(p=0,505)	21
VPL	3,577	(p=0,077)	0,913	(p=0,353)	**8,427**	***(p<0,05)***	20
dHIPPO	0,993	(p=0,333)	0,036	(p=0,852)	0,045	(p=0,834)	21
vHIPPO	1,805	(p=0,197)	2,000	(p=0,175)	2,573	(p=0,127)	21
PAG	0,007	(p=0,933)	1,506	(p=0,238)	0,018	(p=0,896)	20
DR	4,025	(p=0,061)	0,036	(p=0,851)	0,066	(p=0,800)	21
VTA	1,662	(p=0,215)	0,031	(p=0,862)	**4,754**	***(p<0,05)***	21
SN	0,037	(p=0,849)	0,199	(p=0,661)	1,045	(p=0,321)	21
LC	0,173	(p=0,683)	0,309	(p=0,586)	0,300	(p=0,591)	20
LPB	0,185	(p=0,673)	0,240	(p=0,631)	2,034	(p=0,173)	20

Table 7: Two-way ANOVA analysis results for 5HIAA in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Significant interaction between the two factors was detected in PFC, VPL and VTA. All significant values are written in italics in the table.

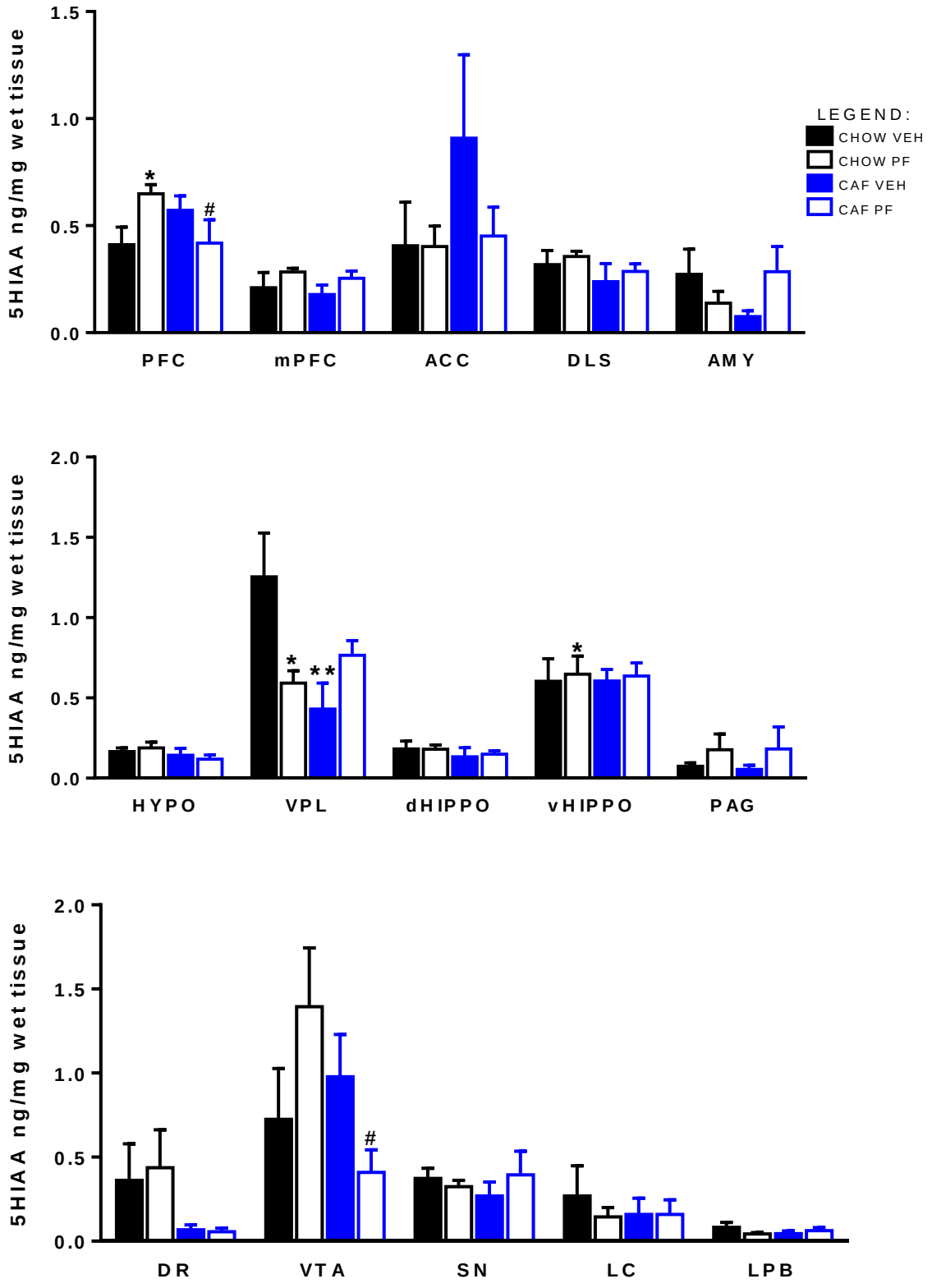

Fig.33: 5HIAA concentration values for each area expressed in ng/mg of wet tissue. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; **p<0,01 vs CHOW VEH; $^{\#}$p<0,05 vs CHOW PF (Tukey's post-hoc test for between groups comparisons).

5HT turnover ratio was influenced by diet withdrawal: animals abstinent from CAFETERIA diet (CAF VEH) presented a significant decrease in LPB when compared to the control group (CHOW VEH). No significant variations were caused by the pharmacological treatment per se in CHOW-fed animals; however, the administration of PF-3845 in CAF PF animals was able to significantly reduce the turnover ratio in HYPO and SN in these animals if compared to the CHOW PF ones.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 8, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 34.

Two-way ANOVA for 5HT turnover in the different areas

	F diet		F treatment		F interaction		df
PFC	0,264	(p=0,614)	0,648	(p=0,432)	1,276	(p=0,274)	21
mPFC	1,180	(p=0,292)	2,089	(p=0,167)	0,807	(p=0,382)	21
ACC	0,520	(p=0,481)	0,515	(p=0,483)	0,008	(p=0,930)	20
DLS	1,960	(p=0,181)	1,228	(p=0,284)	1,635	(p=0,219)	20
AMY	1,077	(p=0,314)	0,128	(p=0,725)	1,492	(p=0,239)	21
HIPO	1,746	(p=0,204)	0,672	(p=0,424)	3,866	(p=0,066)	21
VPL	0,107	(p=0,747)	1,248	(p=0,280)	0,715	(p=0,410)	20
dHYPPO	4,222	(p=0,056)	0,173	(p=0,682)	0,010	(p=0,923)	21
vHYPPO	0,888	(p=0,359)	1,111	(p=0,307)	1,506	(p=0,236)	21
PAG	0,985	(p=0,336)	0,002	(p=0,968)	0,392	(p=0,540)	20
DR	0,135	(p=0,718)	1,639	(p=0,218)	0,584	(p=0,455)	21
VTA	0,141	(p=0,712)	0,122	(p=0,731)	1,591	(p=0,224)	21
SN	2,207	(p=0,156)	0,010	(p=0,923)	2,962	(p=0,103)	21
LC	0,202	(p=0,659)	0,001	(p=0,976)	0,567	(p=0,462)	20
LPB	***4,610***	***(p<0,05)***	0,062	(p=0,807)	1,323	(p=0,267)	20

Table 8: Two-way ANOVA analysis results for 5HT turnover ratio in the various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Diet is a significant factor LPB; no significant interaction between the two factors was detected. All significant values are written in italics in the table.

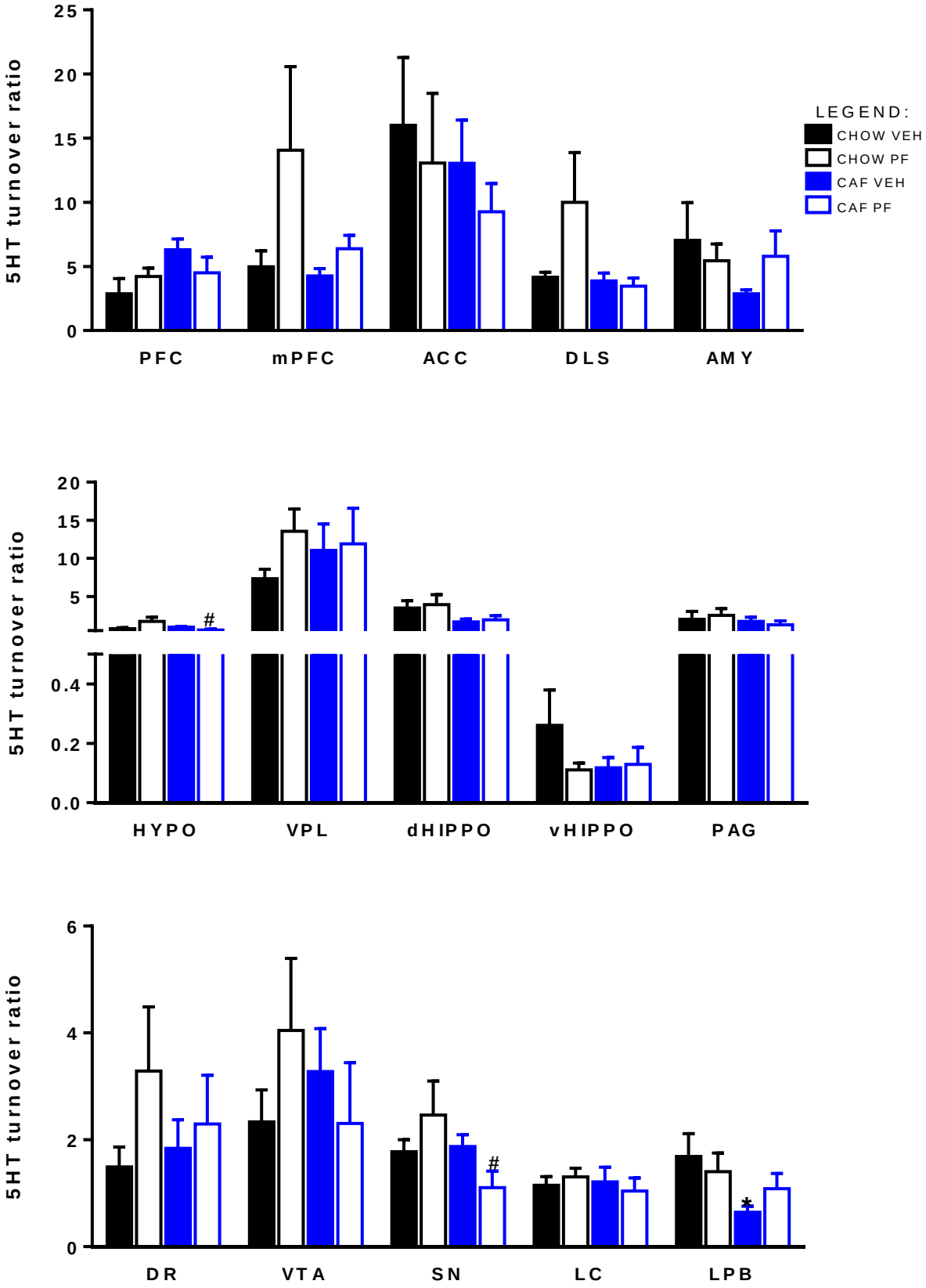

Fig.34: 5HT turnover ratio values for each area expressed in ng/mg of wet tissue. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; #p<0,05 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons).

5. WESTERN BLOT ANALYSIS

After identifying behavioural alterations due to the abstinence from the CAFETERIA diet, and various impairments to the monoaminergic systems caused by the diet withdrawal; we focused our attention on other systems which could be involved in causing the anxiety-like behaviour and depressive-like phenotype. Among them, we focused our interest on the endocannabinoid system and in neuroinflammation.

The endocannabinoid system, as explained in the introduction, has various roles in the central nervous system: it regulates learning, memory and cognition; motor activity; mood tone; appetite and food intake; reward and addiction; neuroprotection; neural development and sleep[170]. Several studies suggest that stress response could affect the endocannabinoid system[266]; and that the endocannabinoid system could have a role in obesity[267] and in addiction[268].

Similarly, also neuroinflammation could be involved: we investigated markers of microglia and neuroinflammation such as IBA-1 and GFAP to discover what are the effects of the withdrawal from CAFETERIA diet and of the pharmacological treatment on the expression of these proteins. It is known that endogenous acylethanolamides (which are increased by the treatment with PF-3845) are important for neural development, synaptic plasticity, reward processing, learning processes, neurogenesis[269,270]; all processes which can be compromised by long-term exposure to high-calorie diets[271]. Furthermore, it has been demonstrated that obesity itself might cause a state of neuroinflammation, with intense activation of astrocyte and microglial response[272,273].

The analysis of the expression of the proteins was executed in the same areas of the HPLC, using the left hemisphere parts, as explained in the material and methods section.

5.1 ENDOCANNABINOID SYSTEM:

We analysed various proteins partaking to the endocannabinoid system. In particular, our attention was focused in cannabinoid receptors type 1 and 2 (respectively CB1 and CB2); enzymes of synthesis and degradation of acylethanolamides (NAPE-PLD, DAGL-alpha, DAGL-beta for synthesis and FAAH, MAGL for degradation).

Western blot analysis of CB1 revealed that diet withdrawal was able to reduce significantly the expression of the receptor in ACC in CAF VEH rats when compared to CHOW VEH ones. Pharmacological treatment, in animals experiencing abstinence from the diet (CAF PF), was significantly able to decrease the expression of CB1 in PFC and HYPO when compared to CAF VEH ones. PF-3845 per se, in CHOW fed animals (CHOW PF), was significantly able to reduce the expression of CB1 in ACC, AMY, HYPO, PFC when compared to vehicle-treated animals (CHOW VEH). Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 9, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 35. Figure 36 shows significant images of western blot analysis.

Two-way ANOVA for CB1 expression in the different areas

	F diet		F treatment		F interaction		df
PFC	1,241	(p=0,277)	**9,924**	***(p<0,01)***	0,056	(p=0,815)	23
mPFC	0,196	(p=0,662)	0,046	(p=0,832)	0,007	(p=0,934)	23
ACC	3,748	(p=0,066)	2,565	(p=0,124)	1,644	(p=0,214)	22
DLS	0,291	(p=0,595)	1,457	(p=0,240)	2,065	(p=0,164)	24
AMY	0,846	(p=0,368)	3,289	(p=0,083)	**7,808**	***(p<0,05)***	23
HYPO	0,397	(p=0,535)	**10,206**	***(p<0,01)***	0,049	(p=0,827)	23
VPL	0,002	(p=0,965)	0,161	(p=0,692)	1,892	(p=0,182)	25
PAG	0,692	(p=0,414)	1,439	(p=0,242)	2,322	(p=0,141)	25
DR	1,112	(p=0,302)	0,783	(p=0,385)	0,495	(p=0,488)	25
VTA	0,076	(p=0,785)	0,374	(p=0,547)	0,581	(p=0,454)	23
SN	2,772	(p=0,110)	0,314	(p=0,581)	0,802	(p=0,380)	23
LC	0,036	(p=0,851)	1,187	(p=0,287)	0,020	(p=0,889)	23
LPB	0,002	(p=0,965)	0,161	(p=0,692)	1,892	(p=0,182)	24
AP	0,996	(p=0,329)	2,131	(p=0,158)	0,528	(p=0,475)	24

Table 9: Two-way ANOVA analysis results for the expression of CB1 in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Treatment is a significant factor in PFC, HYPO; significant interaction between the two factors was detected in AMY. All significant values are written in italics in the table.

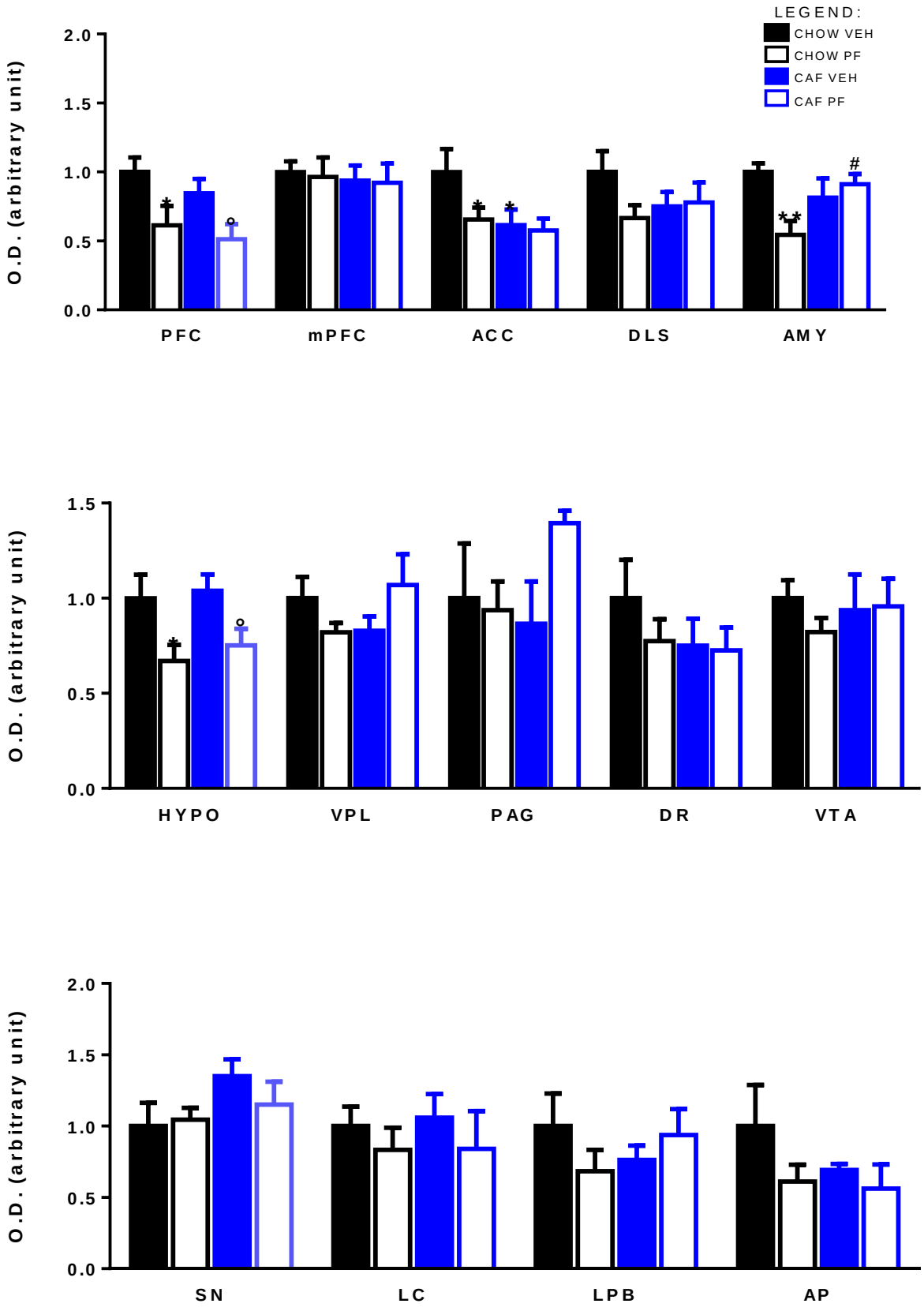

Fig.35: Results of the western blot analysis of CB1 expression; expressed as the protein/adaptin ratio. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; **p<0,01 vs CHOW VEH; °p<0,05 vs CAF VEH; $^{\#}$p<0,05 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons).

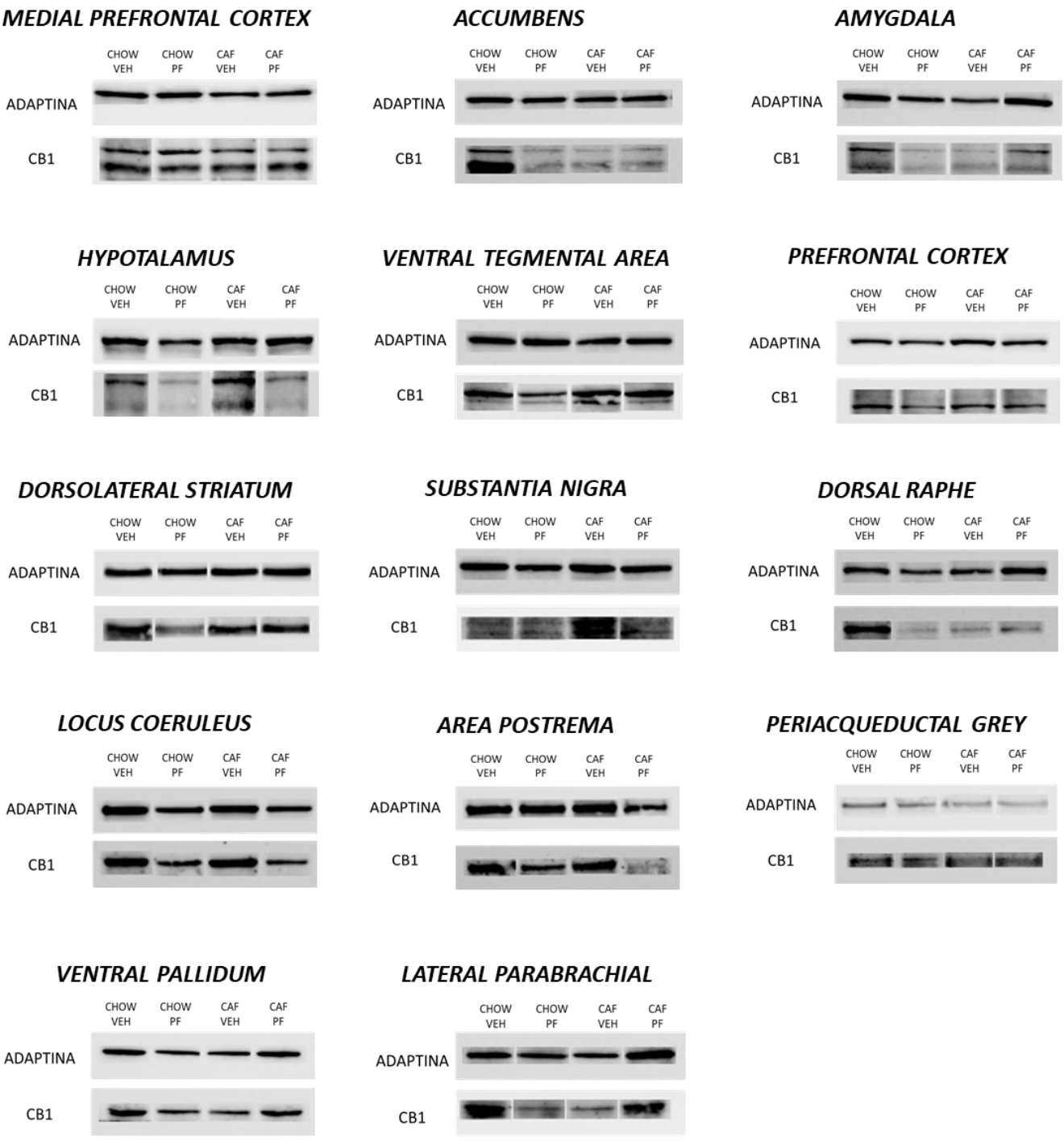

Fig.36: Representative images of western blot analysis of CB1 receptor.

Furthermore, CB2 analysis revealed that diet withdrawal was able to reduce significantly the expression of CB2 in mPFC, ACC and increase it in VTA in CAF VEH animals when compared to CHOW VEH ones. Additionally, pharmacological treatment per se, in CHOW-fed animals (CHOW PF group), was able to significantly increase the expression of CB2 in VTA when compared to CHOW VEH. In PFC, animals abstinent from CAF diet and treated with PF-3845 (CAF PF group) displayed a significant increment of the expression of CB2 when compared both to CAF VEH and CHOW PF animals.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 10, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 37. Figure 38 shows significant images of western blot analysis.

Two-way ANOVA for CB2 expression in the different areas

	F diet		F treatment		F interaction		df
PFC	0,977	(p=0,334)	0,212	(p=0,650)	**7,952**	***(p<0,01)***	23
mPFC	**12,005**	***(p<0,01)***	0,310	(p=0,583)	4,171	(p=0,053)	23
ACC	**12,169**	***(p<0,01)***	1,352	(p=0,258)	0,666	(p=0,424)	22
DLS	0,001	(p=0,975)	0,367	(p=0,551)	2,610	(p=0,120)	24
AMY	0,018	(p=0,894)	0,432	(p=0,518)	0,187	(p=0,670)	23
HYPO	2,368	(p=0,138)	0,542	(p=0,469)	0,001	(p=0,975)	23
VPL	0,161	(p=0,692)	0,726	(p=0,403)	0,144	(p=0,708)	25
DR	0,206	(p=0,654)	0,589	(p=0,450)	3,642	(p=0,068)	25
VTA	**9,429**	***(p<0,01)***	2,945	(p=0,100)	3,271	(p=0,084)	23
SN	0,318	(p=0,579)	0,870	(p=0,361)	0,222	(p=0,642)	23
LC	0,062	(p=0,805)	**4,671**	***(p<0,05)***	0,148	(p=0,704)	23

Table 10: Two-way ANOVA analysis results for the expression of CB2 in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Diet is a significant factor in mPFC, ACC, VTA; treatment in LC. Significant interaction between the two factors was detected in PFC. All significant values are written in italics in the table.

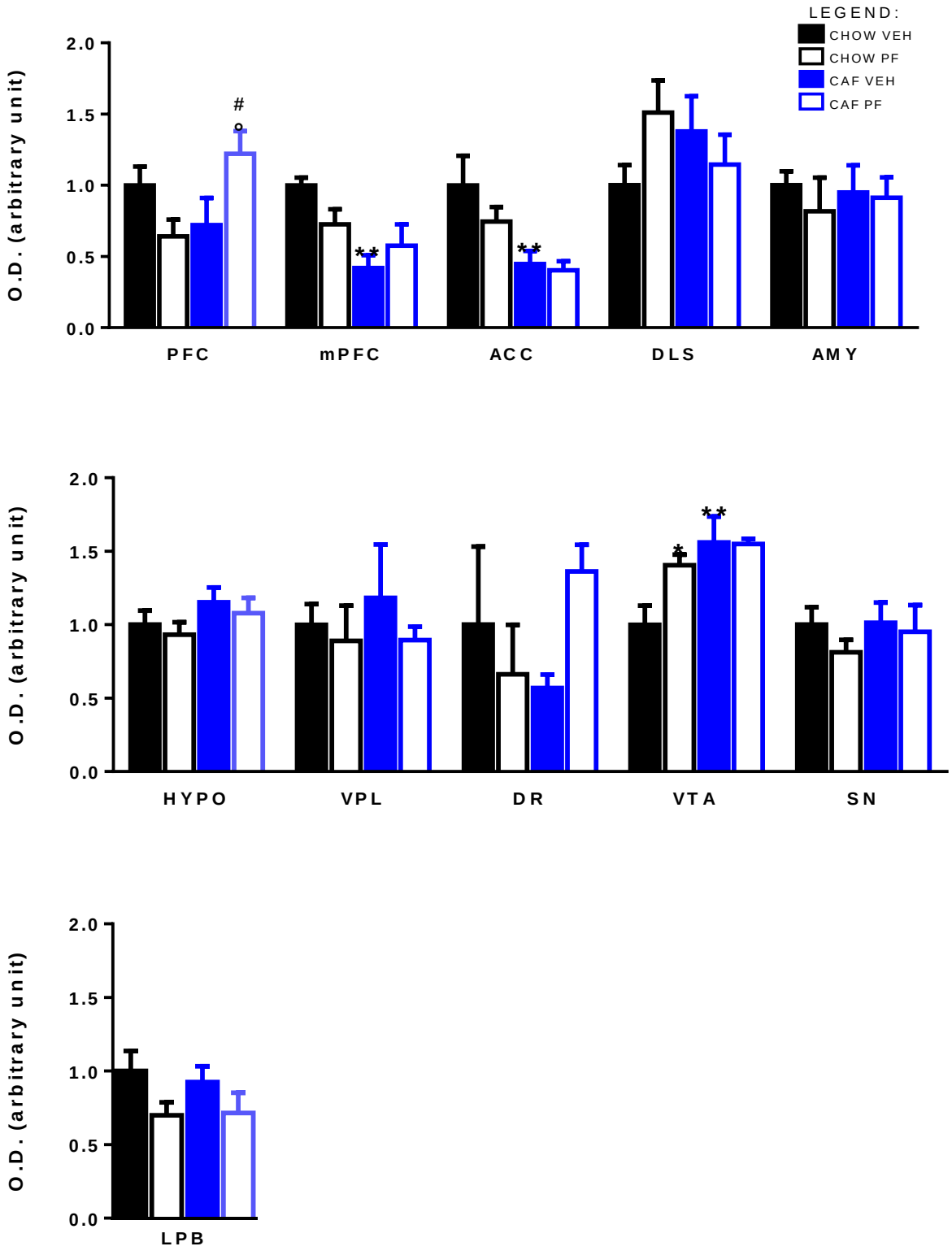

Fig.37: Results of the western blot analysis of CB2 expression; expressed as the protein/adaptin ratio. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; **p<0,01 vs CHOW VEH; °p<0,05 vs CAF VEH; $^{\#}$p<0,05 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons).

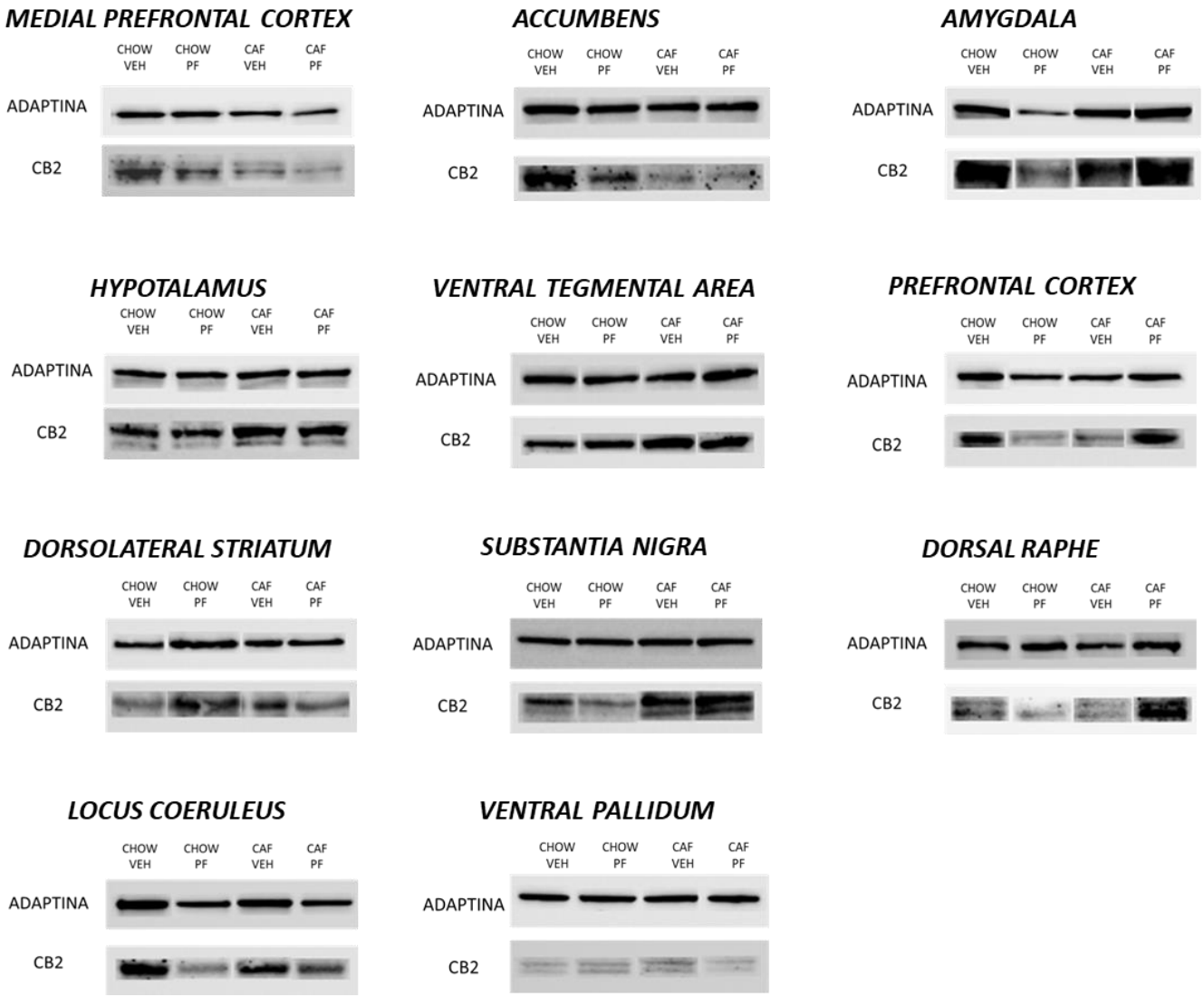

Fig.38: Representative images of western blot analysis of CB2 receptor.

Similarly to all these variations in the expression of cannabinoid receptors, also enzymes of degradation were affected by diet withdrawal and pharmacological treatment. In particular, the expression of the enzyme FAAH was significantly increased by diet withdrawal in VTA in CAF VEH animals when compared to CHOW VEH ones.

PF-3845, when administered in CAF rats (CAF PF group), was able to significantly reduce the expression of FAAH in VTA and increase it in the mPFC when compared to vehicle-treated animals (CAF VEH). Differently, in VPL and LPB, CAF PF animals displayed a significant increase of the expression of FAAH when compared with CHOW PF animals.

When the same treatment was administered in CHOW-fed animals (CHOW PF group), it caused a significant increase of the expression of FAAH in ACC, PAG, VTA and a significant decrease in DR in respect to vehicle administration (CHOW VEH ones).

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 14, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 39. Figure 40 shows significant images of western blot analysis.

Two-way ANOVA for FAAH expression in the different areas

	F diet		F treatment		F interaction		df
PFC	2,182	(p=0,154)	0,101	(p=0,754)	1,467	(p=0,239)	23
mPFC	0,030	(p=0,864)	**7,986**	***(p<0,01)***	0,112	(p=0,741)	23
ACC	**10,669**	***(p<0,01)***	**15,742**	***(p<0,001)***	1,995	(p=0,172)	22
DLS	**10,420**	***(p<0,01)***	0,915	(p=0,349)	0,318	(p=0,578)	24
AMY	1,453	(p=0,241)	0,000	(p=0,992)	1,516	(p=0,231)	23
HYPO	0,009	(p=0,952)	1,618	(p=0,217)	0,901	(p=0,353)	23
VPL	**5,928**	***(p<0,05)***	3,457	(p=0,075)	2,302	(p=0,142)	25
PAG	0,414	(p=0,526)	**5,627**	***(p<0,05)***	0,371	(p=0,548)	25
DR	0,928	(p=0,345)	0,034	(p=0,855)	**8,003**	***(p<0,01)***	25
VTA	**4,953**	***(p<0,05)***	0,086	(p=0,772)	**10,663**	***(p<0,01)***	23
SN	0,261	(p=0,615)	1,090	(p=0,307)	0,754	(p=0,395)	23
LC	0,102	(p=0,752)	0,087	(p=0,077)	0,478	(p=0,497)	23
LPB	2,741	(p=0,111)	0,003	(p=0,957)	3,454	(p=0,076)	24
AP	0,544	(p=0,468)	0,001	(p=0,975)	0,949	(p=0,340)	24

Table 14: Two-way ANOVA analysis results for the expression of FAAH in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Diet is a significant factor in ACC, DLS, VPL, VTA; treatment is a significant factor in mPFC, ACC, PAG. A significant interaction between the two factors was detected in VTA, DR. All significant values are written in italics in the table.

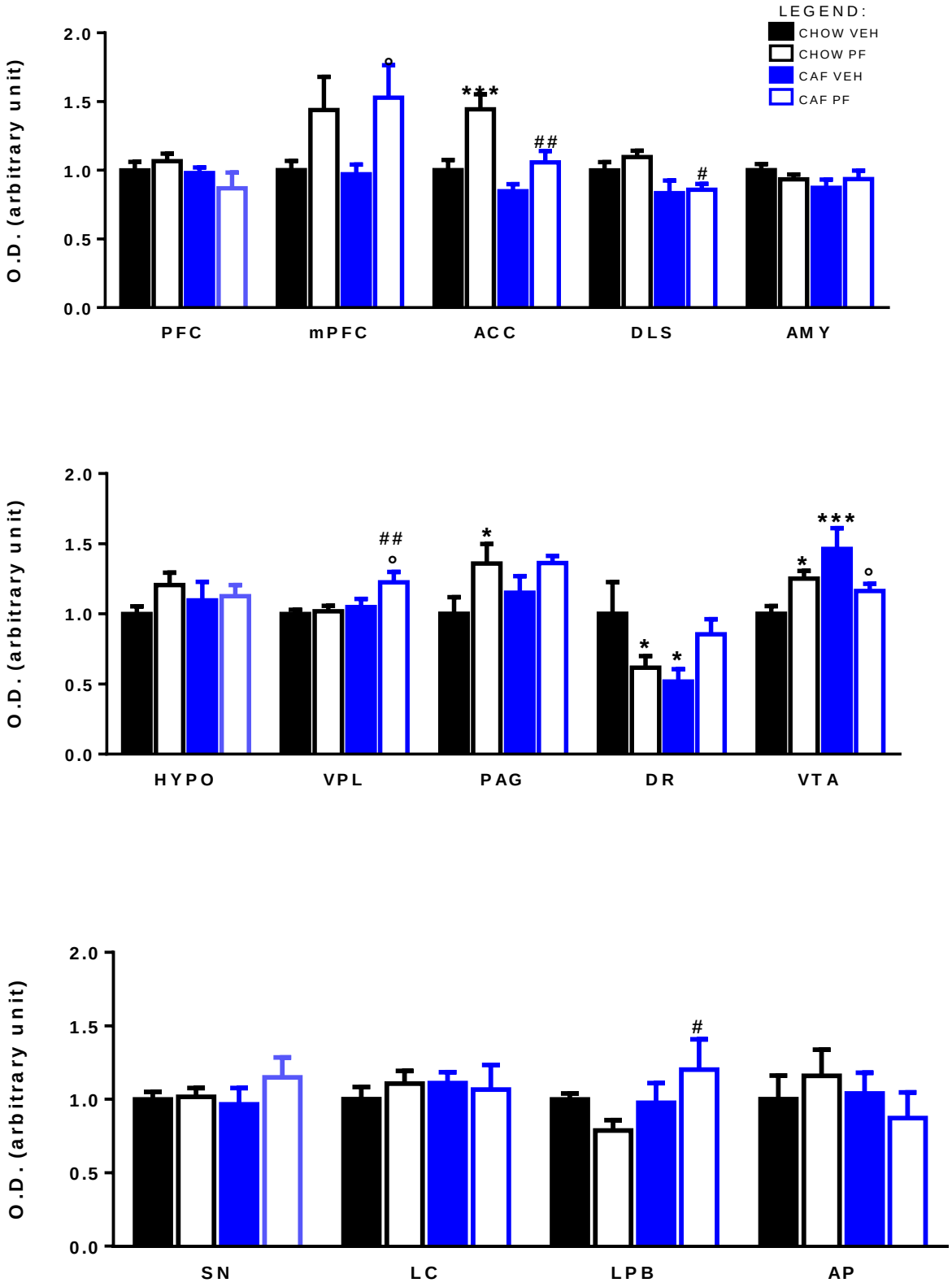

Fig.39: Results of the western blot analysis of FAAH expression; expressed as the protein/adaptin ratio. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; ***p<0,001 vs CHOW VEH; °p<0,05 vs CAF VEH; $^{\#}$p<0,05 vs CHOW PF; $^{\#\#}$p<0,01 vs CHOW PF (Tukey's post-hoc test for between groups comparisons).

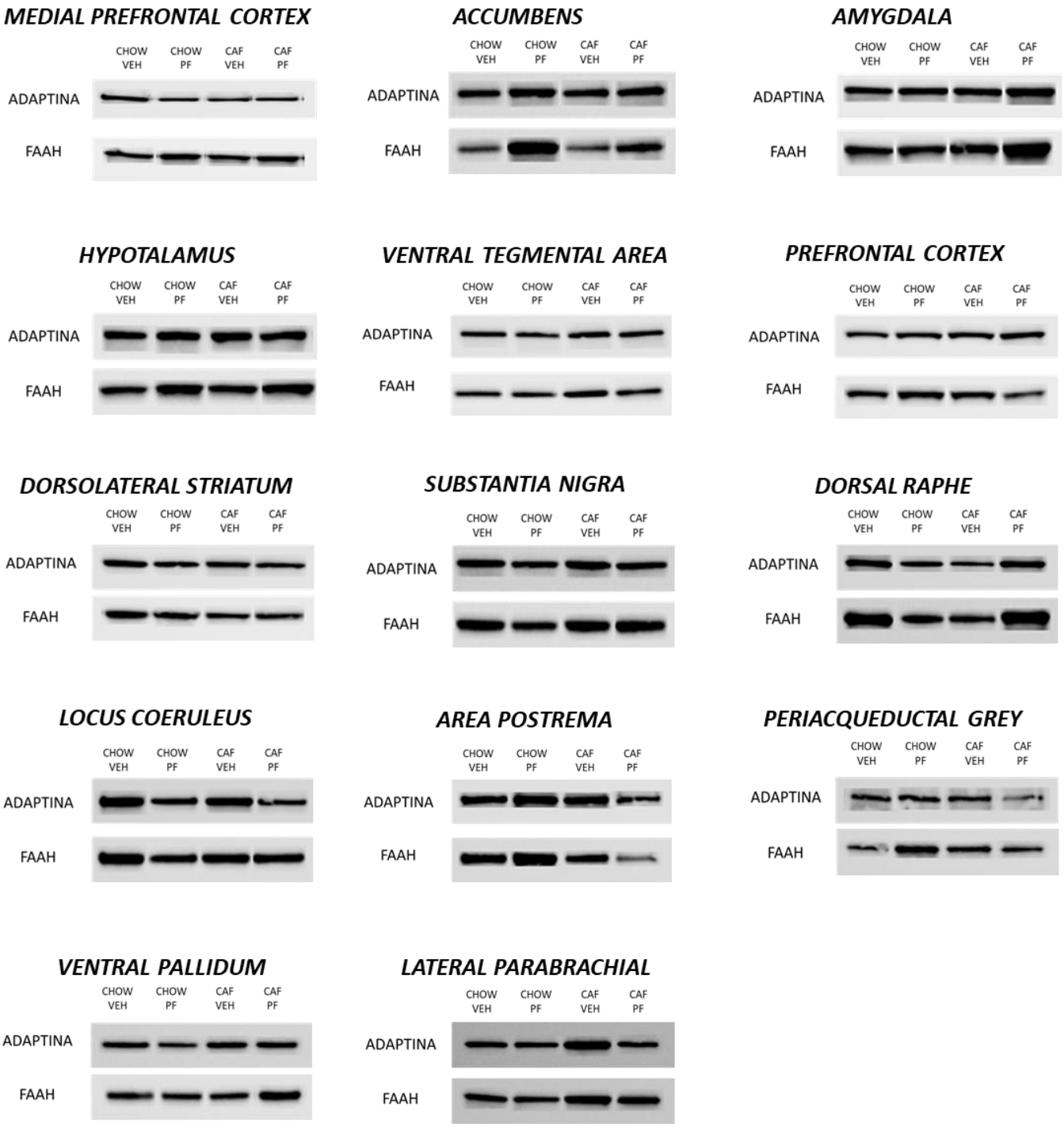

Fig.40: Representative images of western blot analysis of FAAH.

On the other hand, in AMY MAGL expression was significantly reduced in CAF VEH rats when compared to CHOW VEH; PF-3845 administration in CAF PF group was able to significantly increase this expression compared to CAF VEH group and restore it to control values. Differently, in DLS MAGL expression was significantly higher in CAF VEH rats; when compared to CHOW VEH. PF-3845 administration in animals abstinent from the diet (CAF PF) reduced the expression of MAGL when compared to CHOW PF group both in DLS and VTA. When PF-3845 was administered in CHOW-fed animals (CHOW PF), it caused a significant increase of the expression of MAGL in VTA and DLS and a decrease in AMY and SN with respect to CHOW VEH group.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 15, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 41. Figure 42 shows significant images of western blot analysis.

Two-way ANOVA for MAGL expression in the different areas

	F diet		F treatment		F interaction		df
PFC	0,059	(p=0,810)	1,296	(p=0,267)	1,976	(p=0,174)	23
mPFC	2,507	(p=0,128)	0,221	(p=0,643)	0,354	(p=0,558)	23
ACC	3,607	(p=0,071)	0,134	(p=0,718)	0,602	(p=0,446)	22
DLS	0,536	(p=0,471)	**5,744**	***(p<0,05)***	**21,169**	***(p<0,001)***	24
AMY	1,296	(p=0,267)	0,000	(p=0,992)	**10,899**	***(p<0,01)***	23
HYPO	0,320	(p=0,577)	0,000	(p=0,992)	0,016	(p=0,900)	23
VTA	0,721	(p=0,405)	0,969	(p=0,337)	4,076	(p=0,055)	23
SN	0,001	(p=0,975)	2,958	(p=0,099)	2,444	(p=0,132)	23
LPB	1,669	(p=0,210)	0,120	(p=0,732)	0,000	(p=0,992)	24

Table 15: Two-way ANOVA analysis results for the expression of MAGL in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Treatment was a significant factor in DLS. Significant interaction between the two factors was detected in DLS, AMY. All significant values are written in italics in the table.

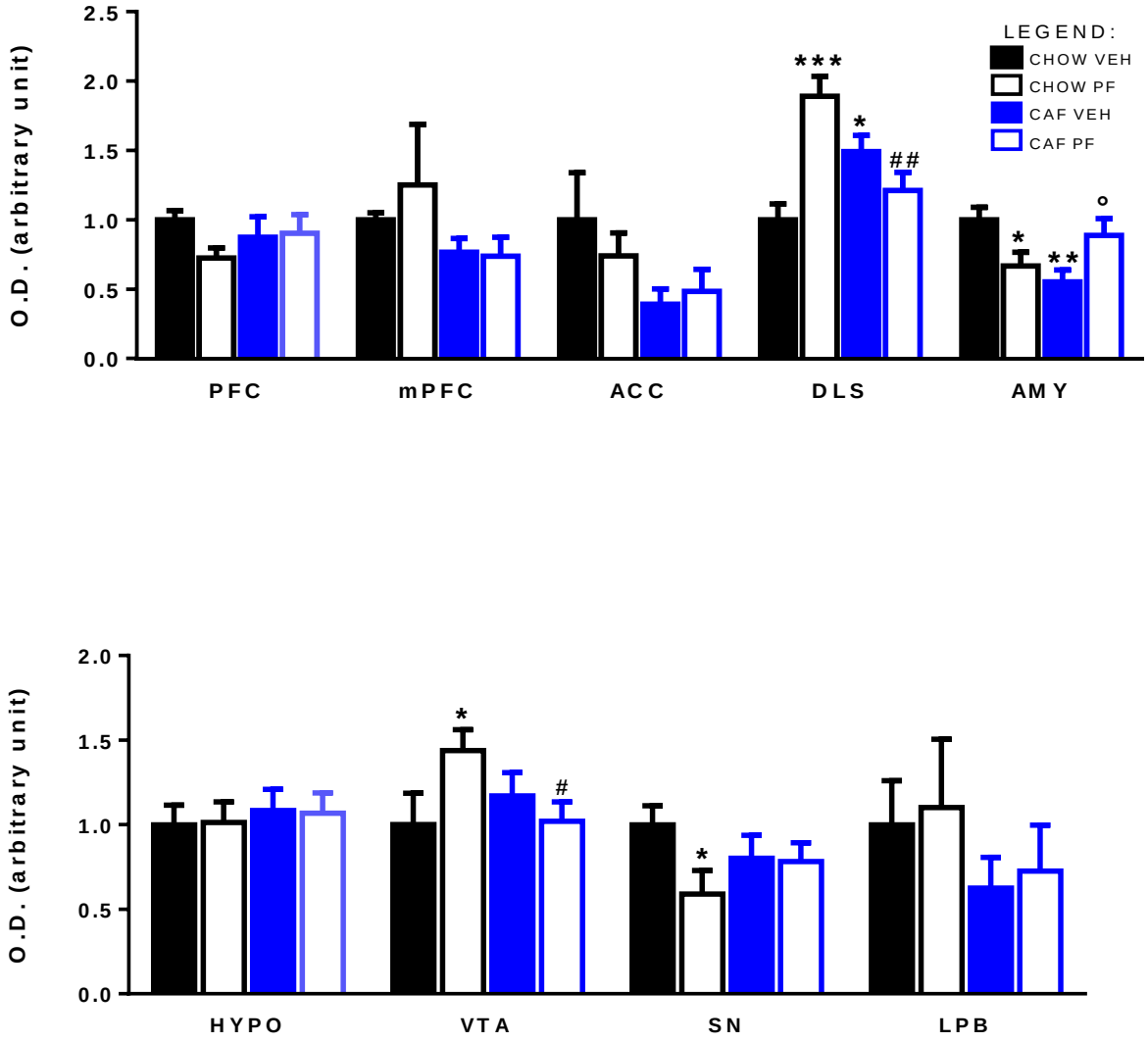

Fig.41: Results of the western blot analysis of MAGL expression; expressed as the protein/adaptin ratio. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; **p<0,01 vs CHOW VEH; ***p<0,001 vs CHOW VEH; °p<0,05 vs CAF VEH; $^{\#}$p<0,05 vs CHOW PF; $^{\#\#}$p<0,01 vs CHOW PF (Tukey's post-hoc test for between groups comparisons).

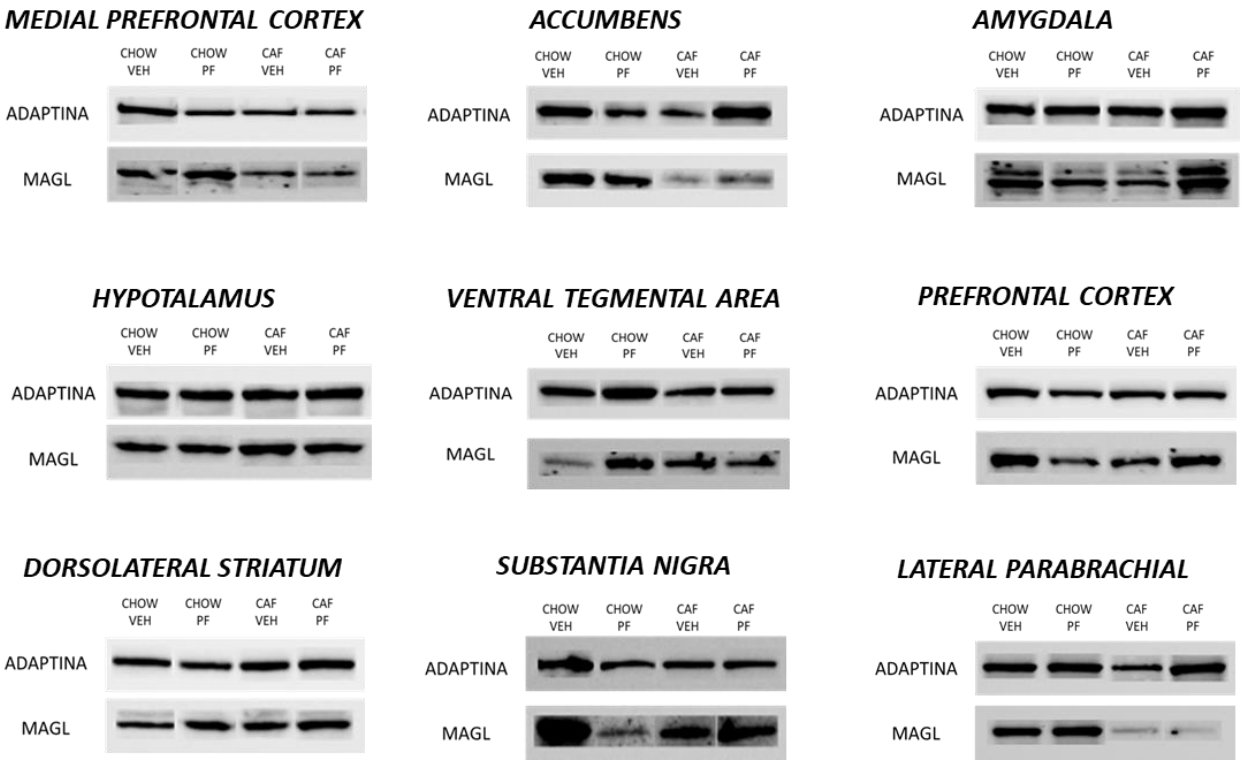

Fig.42: Representative images of western blot analysis of MAGL.

Also, NAPE-PLD expression was affected by CAFETERIA diet abstinence: animals experiencing diet withdrawal (CAF VEH) presented significant reduction of the expression of the protein in mPFC, AMY, SN and LPB when compared to CHOW VEH. In CAF PF animals, the pharmacological treatment was able to significantly reduce the expression of NAPE-PLD in VTA, compared to vehicle-treated ones (CAF VEH). PF-3845 administration per se, in CHOW-fed animals (CHOW PF), was able to significantly decrease the expression of the protein in SN, when compared to vehicle-treated ones (CHOW VEH). In DR, animals abstinent from CAF diet and treated with PF-3845 (CAF PF) displayed a significant increment of the expression of NAPE-PLD when compared both to CAF VEH and CHOW PF animals; whereas in LC they presented a significant decrease of the expression of the protein when compared both to CAF VEH and CHOW PF animals.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 11, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 43. Figure 44 shows significant images of western blot analysis.

Two-way ANOVA for NAPE PLD expression in the different areas

	F diet		F treatment		F interaction		df
PFC	0,130	(p=0,722)	0,162	(p=0,691)	3,706	(p=0,067)	23
mPFC	**8,309**	***(p<0,01)***	1,320	(p=0,263)	1,478	(p=0,237)	23
ACC	0,559	(p=0,463)	0,927	(p=0,347)	0,001	(p=0,975)	22
DLS	1,716	(p=0,204)	1,203	(p=0,285)	1,104	(p=0,305)	24
AMY	0,938	(p=0,343)	0,398	(p=0,535)	3,526	(p=0,074)	23
HYPO	2,485	(p=0,129)	**10,552**	***(p<0,01)***	0,981	(p=0,333)	23
PAG	2,277	(p=0,144)	**8,271**	***(p<0,01)***	0,016	(p=0,900)	25
DR	2,189	(p=0,152)	**5,152**	***(p<0,05)***	2,986	(p=0,096)	25
VTA	0,255	(p=0,619)	3,250	(p=0,085)	4,284	(p=0,050)	23
SN	**7,165**	***(p<0,05)***	**4,955**	***(p<0,05)***	**4,936**	***(p<0,05)***	23
LC	**5,672**	***(p<0,05)***	**5,218**	***(p<0,05)***	1,505	(p=0,232)	23
LPB	**4,621**	***(p<0,05)***	1,718	(p=0,202)	1,024	(p=0,322)	24

Table 11: Two-way ANOVA analysis results for the expression of NAPE-PLD in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Diet is a significant factor in mPFC, SN, LC, LPB; treatment is a significant factor in HYPO, PAG, DR, SN, LC. A significant interaction between the two factors was detected in SN. All significant values are written in italics in the table.

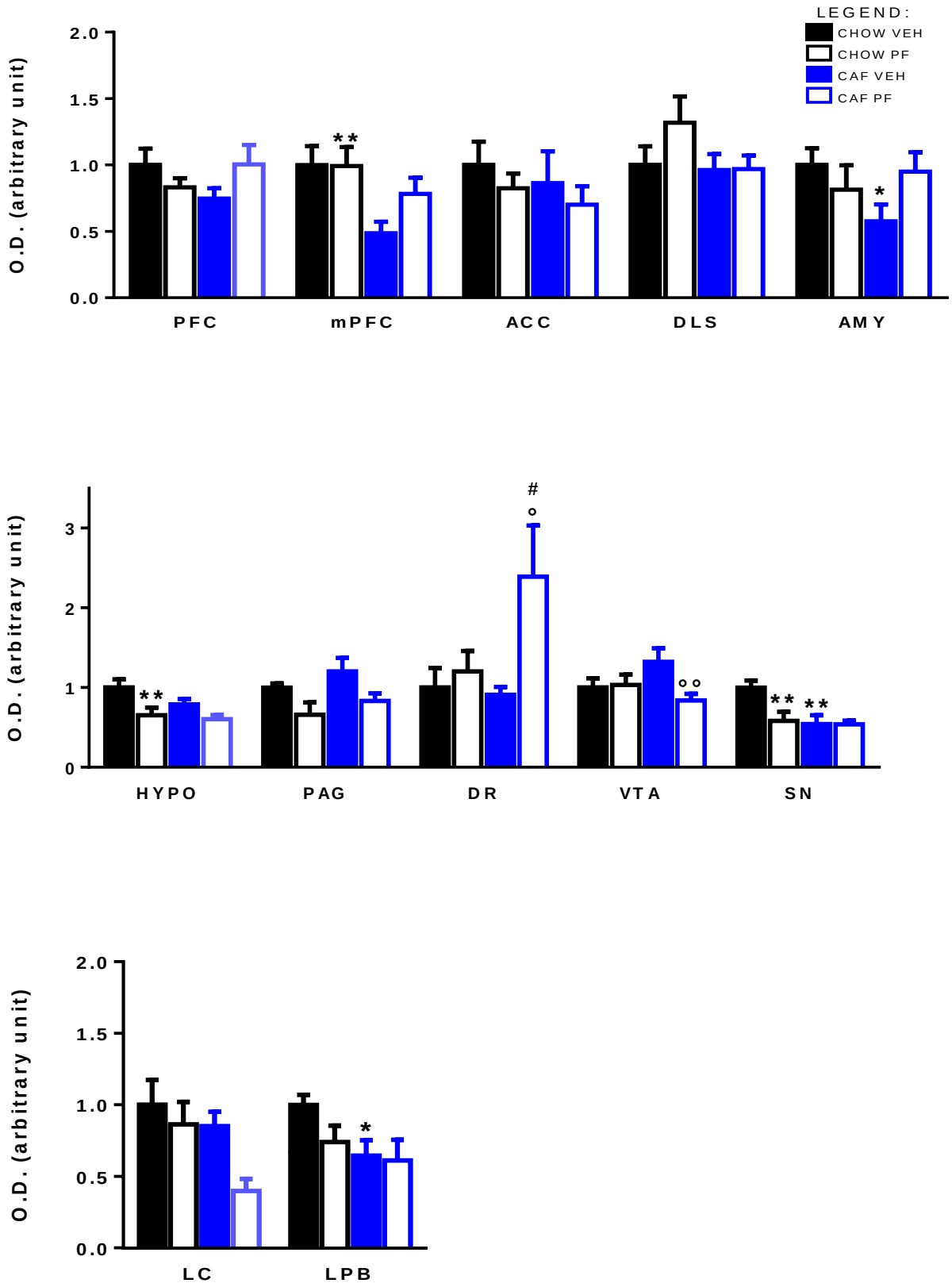

Fig.43: Results of the western blot analysis of NAPE-PLD expression expressed as the protein/adaptin ratio. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; **p<0,01 vs CHOW VEH; °p<0,05 vs CAF VEH; °°p<0,01 vs CAF VEH; $^{\#}$p<0,05 vs CHOW PF (Tukey's post-hoc test for between groups comparisons).

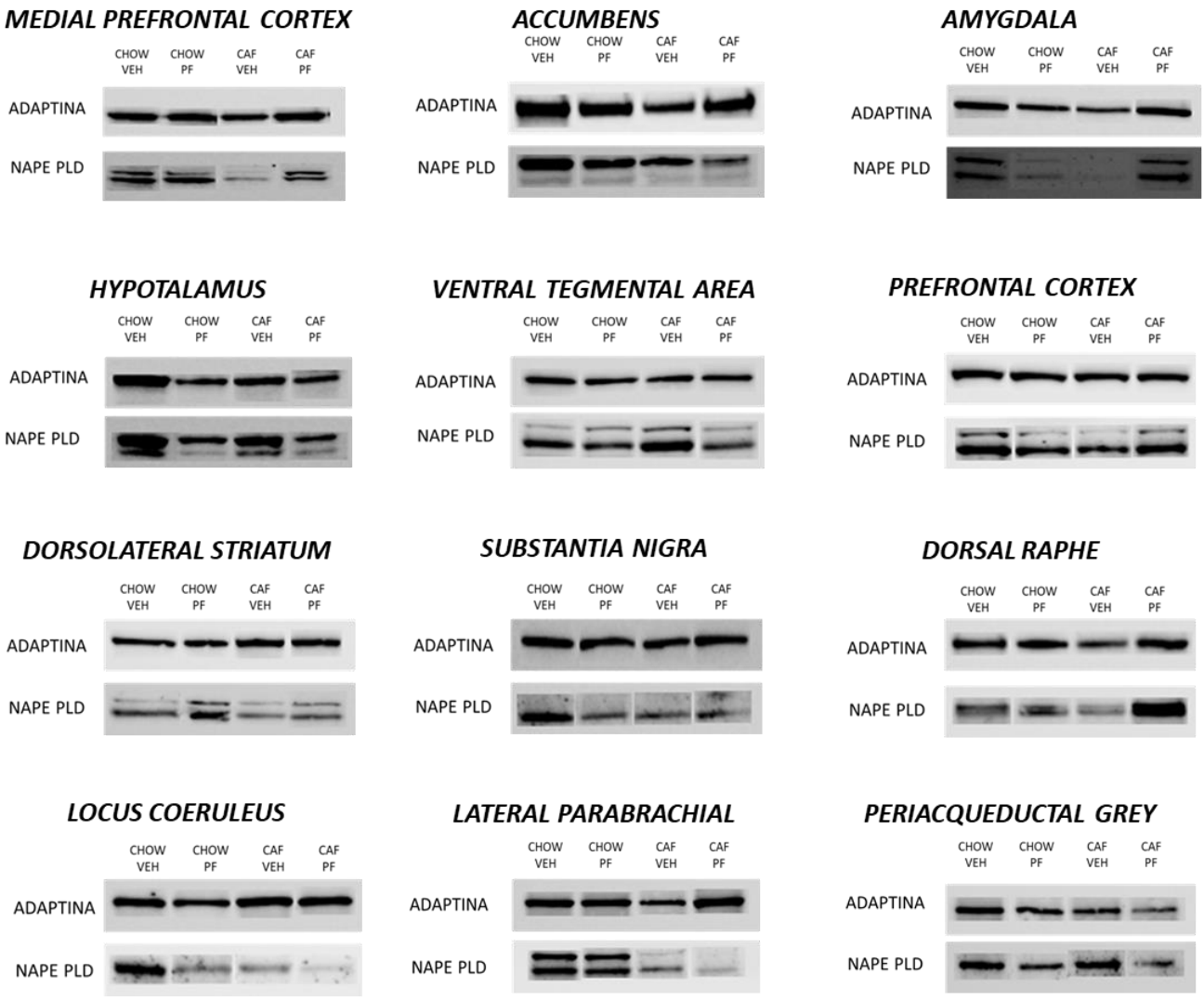

Fig.44: Representative images of western blot analysis of NAPE PLD.

The expression of DAGL-alpha was also analysed: diet withdrawal caused a significant decrease in the expression of the protein in ACC and AMY in CAF VEH animals when compared to CHOW VEH ones. Pharmacologic treatment was effective in CAF PF animals in increasing the expression of DAGL-alpha in AMY and decreasing it in HYPO; compared to vehicle-treated animals (CAF VEH). By the way, PF-3845 per se in CHOW-fed animals (CHOW PF group) significantly decreased the expression of the protein in AMY and LC when compared to vehicle-treated rats (CHOW VEH).

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 12, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 45. Figure 46 shows representative images of western blot analysis.

Two-way ANOVA for DAGL alpha expression in the different areas

	F diet		F treatment		F interaction		df
PFC	0,208	(p=0,653)	0,003	(p=0,957)	0,287	(p=0,598)	23
mPFC	**5,146**	***(p<0,05)***	0,016	(p=0,900)	0,227	(p=0,638)	23
ACC	**16,378**	***(p<0,001)***	0,000	(p=0,992)	0,341	(p=0,565)	22
DLS	0,000	(p=0,992)	**4,625**	***(p<0,05)***	0,000	(p=0,992)	24
AMY	2,387	(p=0,136)	0,048	(p=0,829)	**9,004**	***(p<0,001)***	23
HYPO	0,004	(p=0,950)	1,105	(p=0,305)	**4,754**	***(p<0,05)***	23
VPL	1,124	(p=0,300)	1,940	(p=0,176)	0,264	(p=0,612)	25
PAG	0,648	(p=0,429)	0,315	(p=0,580)	0,723	(p=0,404)	25
DR	0,034	(p=0,855)	0,120	(p=0,732)	2,035	(p=0,167)	25
VTA	0,320	(p=0,577)	1,016	(p=0,324)	0,473	(p=0,499)	23
SN	0,002	(p=0,965)	0,644	(p=0,431)	1,160	(p=0,293)	23
LC	0,029	(p=0,866)	1,033	(p=0,321)	3,970	(p=0,059)	23
AP	0,090	(p=0,767)	0,221	(p=0,643)	0,005	(p=0,944)	24

Table 12: Two-way ANOVA analysis results for the expression of DAGL-alpha in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Diet is a significant factor in mPFC, ACC. Treatment is a significant factor in DLS. Significant interaction between the two factors was detected in AMY and HYPO. All significant values are written in italics in the table.

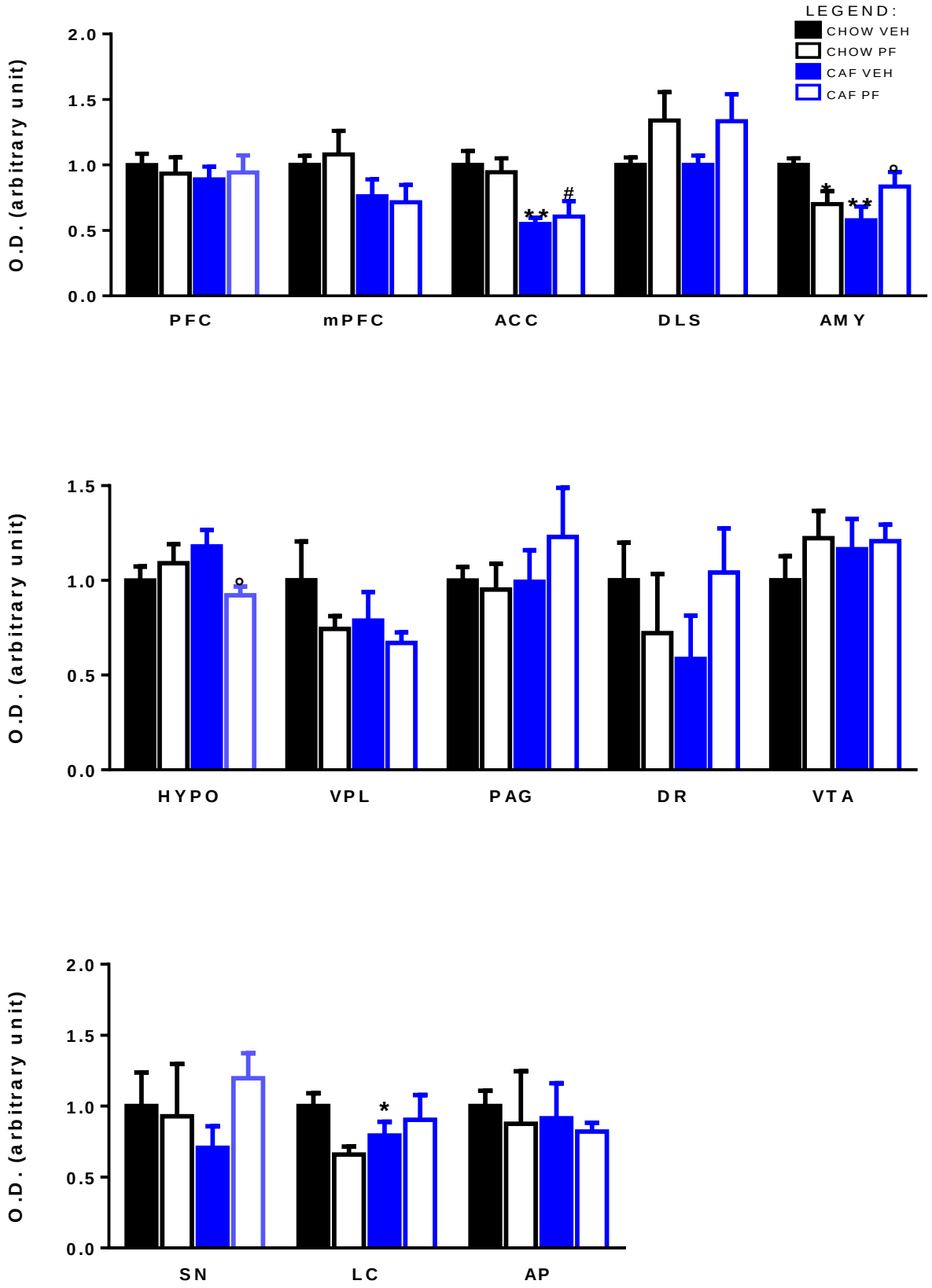

Fig.45: Results of the western blot analysis of DAGL-alpha expression; expressed as the protein/adaptin ratio. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; **p<0,01 vs CHOW VEH; °p<0,05 vs CAF VEH; $^{\#}$p<0,05 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons).

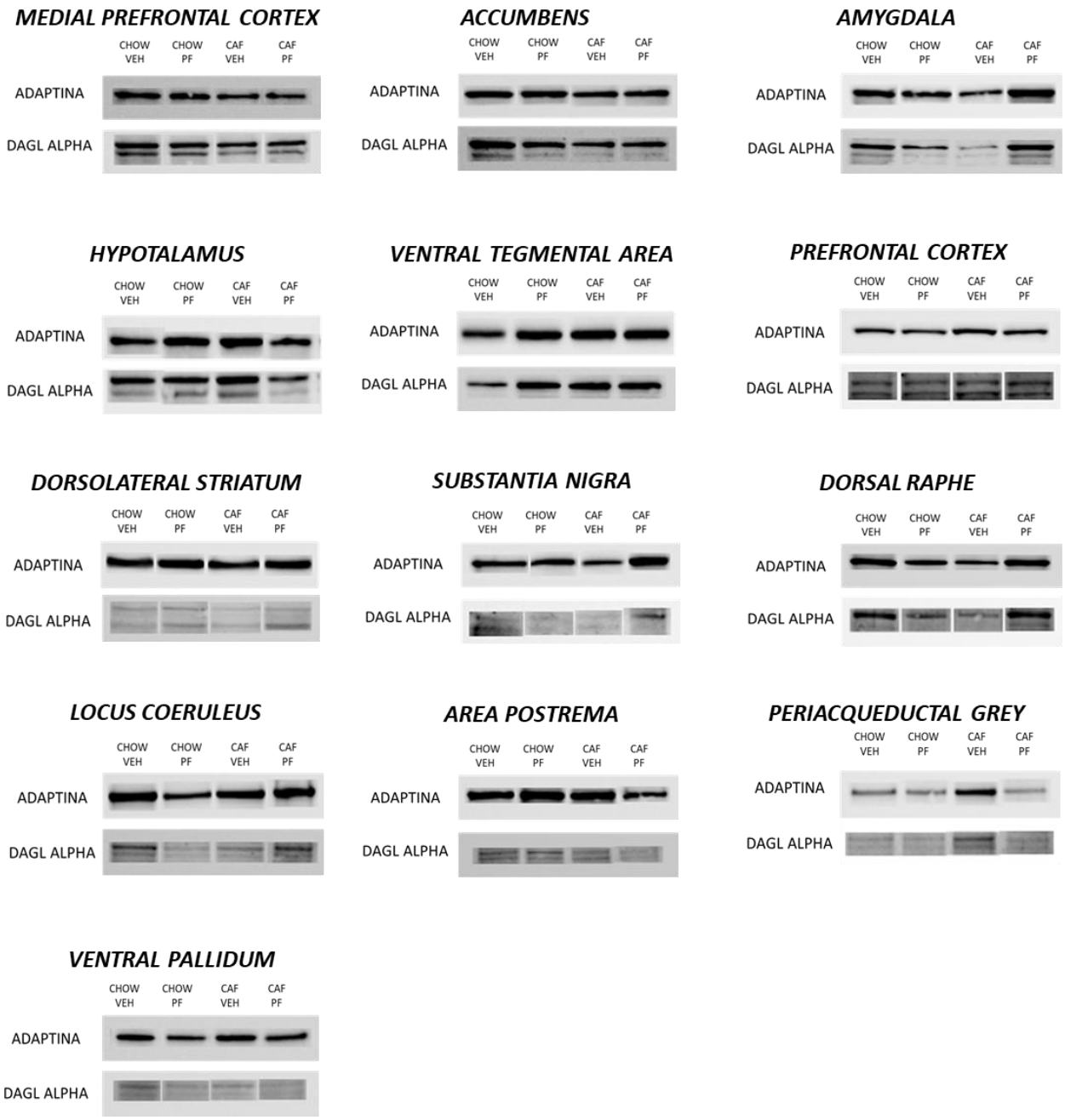

Fig.46: Representative images of western blot analysis of DAGL alpha.

Along with the variations of DAGL-alpha, diet withdrawal affected also the expression of DAGL-beta, in particular in the HYPO and PFC, where we observed a significant decrease of the expression of the protein in CAF VEH animals if compared to control animals (CHOW VEH). Besides, PF-3845 administration was able to reduce significantly the expression of DAGL-beta in CHOW-fed animals (CHOW PF) if compared to vehicle-treated ones (CHOW VEH) in HYPO. Moreover, in DLS, pharmacological treatment per se was able to increase the expression of DAGL-beta in CHOW-fed animals (CHOW PF) compared to vehicle-treated ones (CHOW VEH).

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 13, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 45. Figure 46 shows representative images of western blot analysis.

Two-way ANOVA for DAGL beta expression in the different areas

	F diet		F treatment		F interaction		df
PFC	7,542	(p=0,012)	0,075	(p=0,787)	0,179	(p=0,676)	23
mPFC	3,314	(p=0,082)	0,037	(p=0,849)	0,876	(p=0,359)	23
ACC	0,211	(p=0,651)	0,001	(p=0,975)	0,034	(p=0,855)	22
DLS	1,072	(p=0,311)	2,152	(p=0,156)	3,148	(p=0,089)	24
AMY	4,170	(p=0,053)	3,505	(p=0,074)	2,472	(p=0,130)	23
HYPO	**19,526**	***(p<0,001)***	**7,825**	***(p<0,05)***	3,925	(p=0,060)	23
VPL	0,000	(p=0,992)	1,478	(p=0,236)	0,062	(p=0,805)	25
DR	0,213	(p=0,649)	0,685	(p=0,416)	0,594	(p=0,488)	25
VTA	0,497	(p=0,488)	0,088	(p=0,770)	1,633	(p=0,215)	23
SN	0,020	(p=0,888)	2,722	(p=0,133)	0,537	(p=0,471)	23
LC	1,904	(p=0,181)	0,063	(p=0,804)	0,968	(p=0,336)	23
LPB	0,053	(p=0,820)	0,470	(p=0,499)	0,010	(p=0,921)	24
AP	**11,754**	***(p<0,01)***	1,602	(p=0,218)	0,234	(p=0,633)	24

Table 13: Two-way ANOVA analysis results for the expression of DAGL-beta in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Diet is a significant factor in HYPO, AP; treatment is a significant factor in HYPO. No significant interaction between the two factors was detected. All significant values are written in italics in the table.

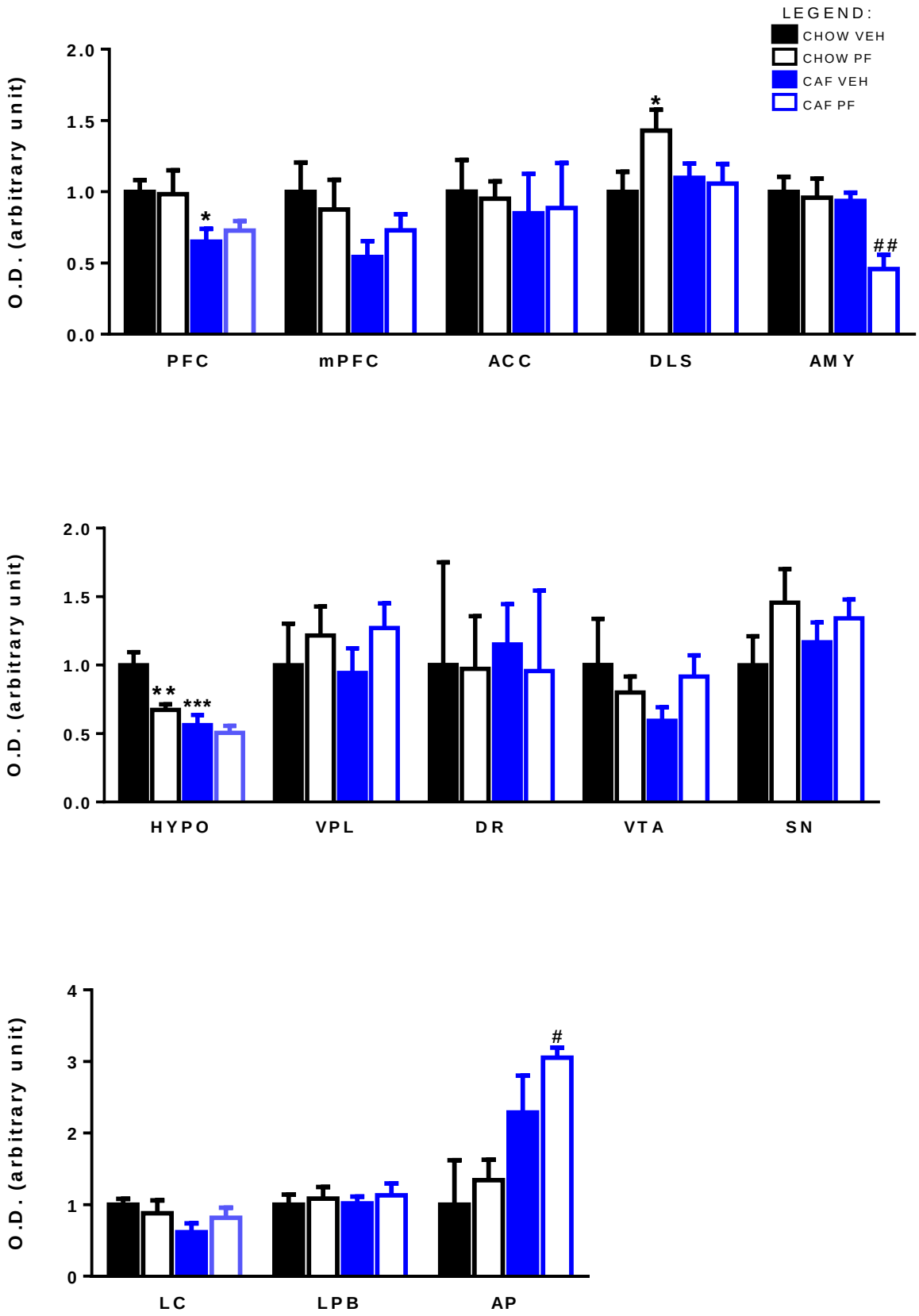

Fig.47: Results of the western blot analysis of DAGL-beta expression; expressed as the protein/adaptin ratio. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; **p<0,01 vs CHOW VEH; ***p<0,001 vs CHOW VEH; $^{\#}$p<0,05 vs CHOW PF; $^{\#\#}$p<0,01 vs CHOW PF (Tukey's post-hoc test for between groups comparisons).

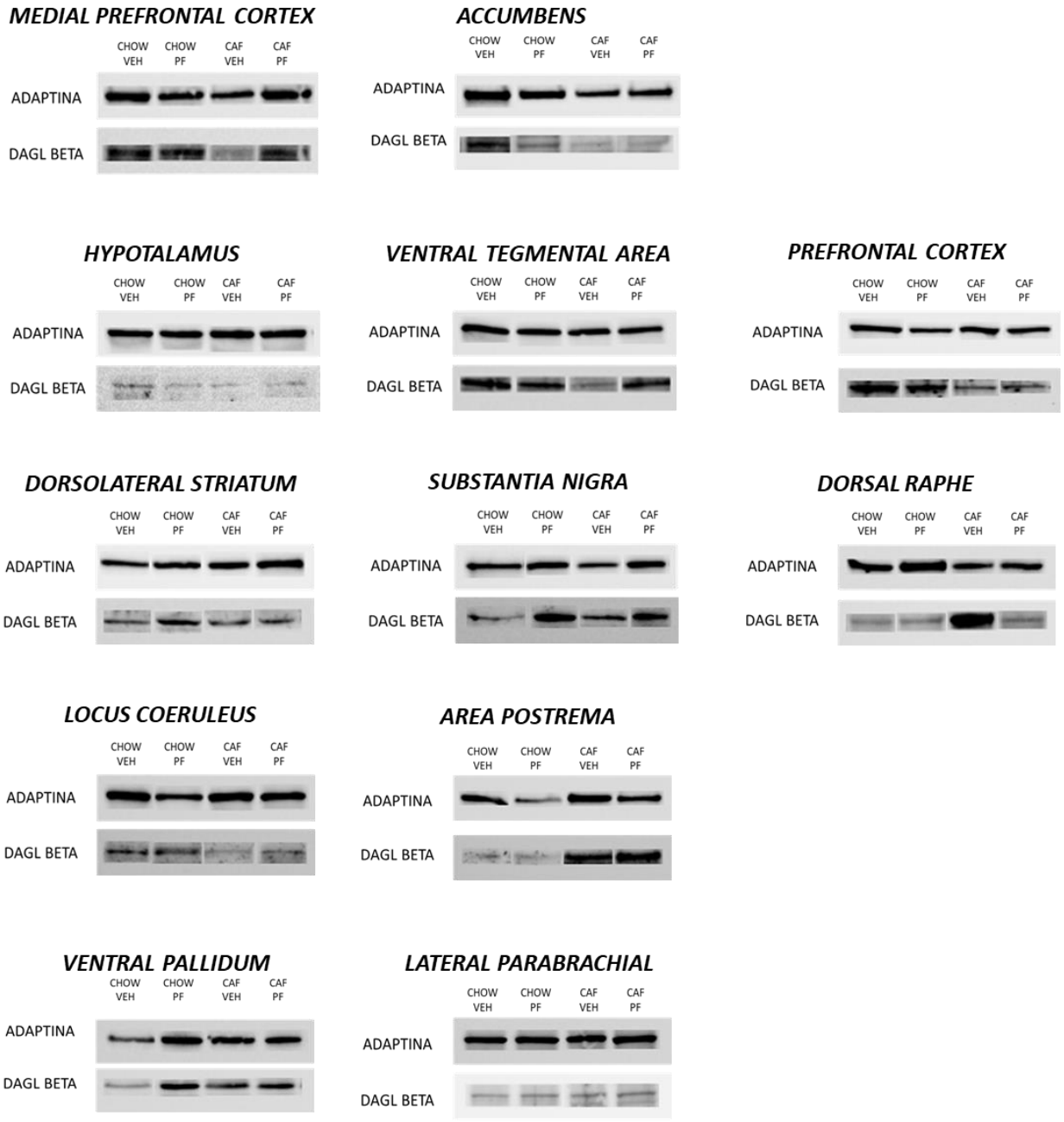

Fig.48: Representative images of western blot analysis of DAGL beta.

5.2 NEUROINFLAMMATORY MARKERS:

To investigate possible neuroinflammation caused by diet withdrawal and a possible protective effect of the pharmacological treatment, we analysed two main proteins involved in neuroinflammation and the activation of microglia; such as GFAP and IBA-1. Moreover, the analysis of the expression of the enzyme COX-2 involved in the production of pro-inflammatory mediators, was performed in HYPO, the brain area more implied in the starting of a neuroinflammatory response. Regarding the expression of GFAP, our analysis revealed that diet withdrawal significantly reduced the expression of the protein in mPFC and DR in CAF VEH animal, if compared to CHOW VEH rats. In CAF rats, the administration of PF-3845 was significantly able to increase the expression of the protein in mPFC, VTA and LPB if compared to vehicle administrated rats. Pharmacological treatment per se in CHOW PF animals was able to decrease GFAP expression in mPFC and DR when compared to CHOW VEH. Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 16, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 49. Figure 50 shows representative images of western blot analysis.

Two-way ANOVA for GFAP expression in the different areas

	F diet		F treatment		F interaction		df
PFC	3,869	(p=0,062)	2,064	(p=0,162)	0,239	(p=0,630)	23
mPFC	**4,348**	***(p<0,05)***	1,015	(p=0,325)	**16,923**	***(p<0,001)***	23
ACC	0,103	(p=0,751)	0,453	(p=0,508)	0,057	(p=0,814)	22
DLS	0,435	(p=0,516)	0,595	(p=0,448)	0,010	(p=0,921)	24
AMY	**4,423**	***(p<0,05)***	**5,739**	***(p<0,05)***	0,087	(p=0,770)	23
HYPO	1,264	(p=0,273)	1,482	(p=0,236)	0,040	(p=0,843)	23
VPL	0,159	(p=0,694)	0,000	(p=0,992)	0,798	(p=0,380)	25
PAG	0,117	(p=0,735)	0,360	(p=0,554)	2,726	(p=0,112)	25
DR	0,447	(p=0,510)	0,635	(p=0,433)	**10,036**	***(p<0,01)***	25
VTA	2,044	(p=0,166)	**8,153**	***(p<0,01)***	0,571	(p=0,458)	23
SN	0,431	(p=0,518)	0,009	(p=0,925)	1,636	(p=0,214)	23
LC	1,216	(p=0,282)	0,307	(p=0,585)	0,084	(p=0,775)	23
LPB	0,001	(p=0,975)	3,949	(p=0,058)	**5,703**	***(p<0,05)***	24
AP	0,912	(p=0,350)	0,027	(p=0,871)	0,389	(p=0,539)	24

Table 16: Two-way ANOVA analysis results for the expression of GFAP in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Diet is a significant factor in mPFC and AMY; treatment is a significant factor in AMY and VTA. A significant interaction between the two factors was detected in mPFC, DR, LPB. All significant values are written in italics in the table.

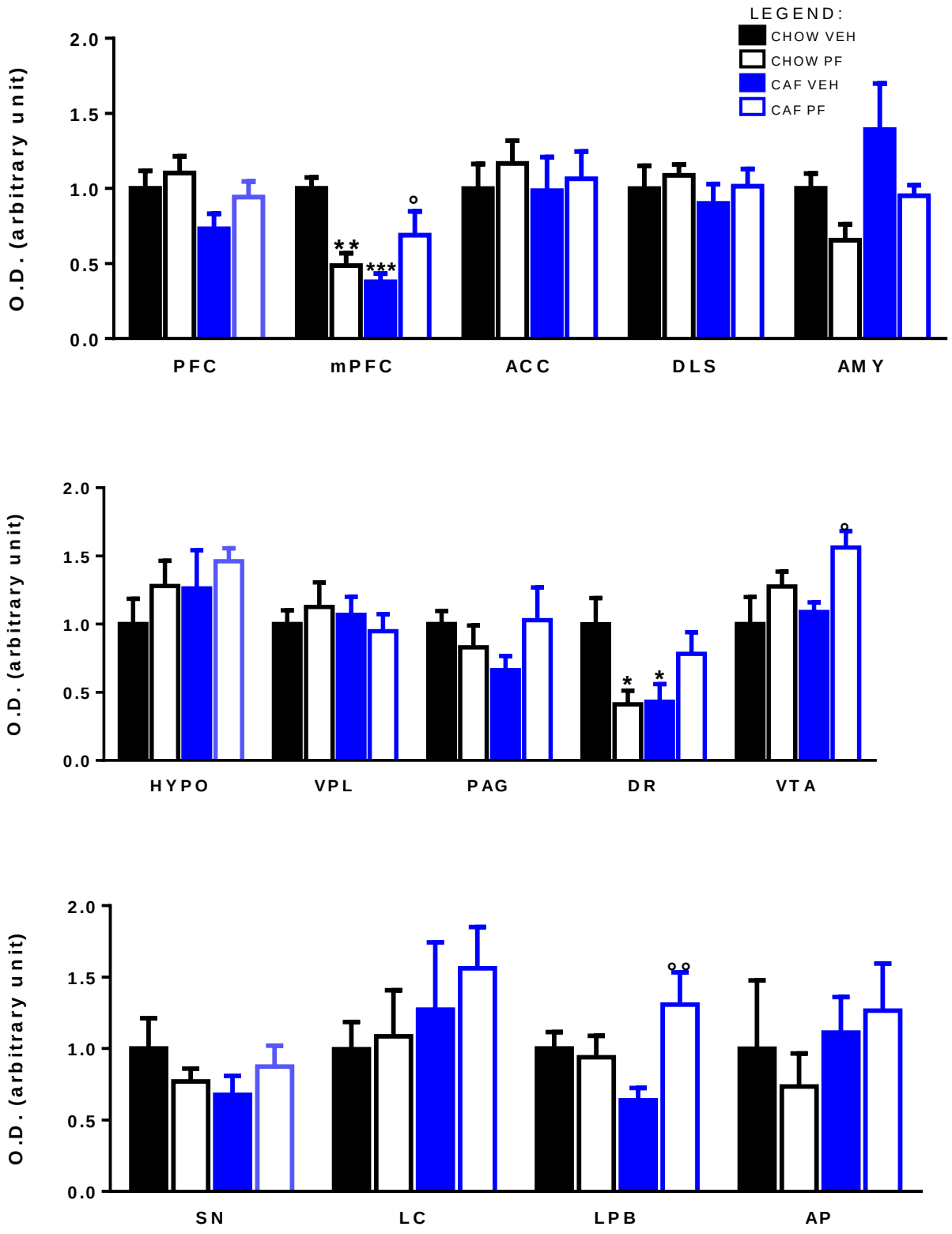

Fig.49: Results of the western blot analysis of GFAP expression; expressed as the protein/adaptin ratio. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; **p<0,01 vs CHOW VEH; ***p<0,001 vs CHOW VEH; °p<0,05 vs CAF VEH; °°p<0,01 vs CAF VEH; $^{\#}$p<0,05 vs CHOW PF; $^{\#\#}$p<0,01 vs CHOW PF (Tukey's post-hoc test for between groups comparisons).

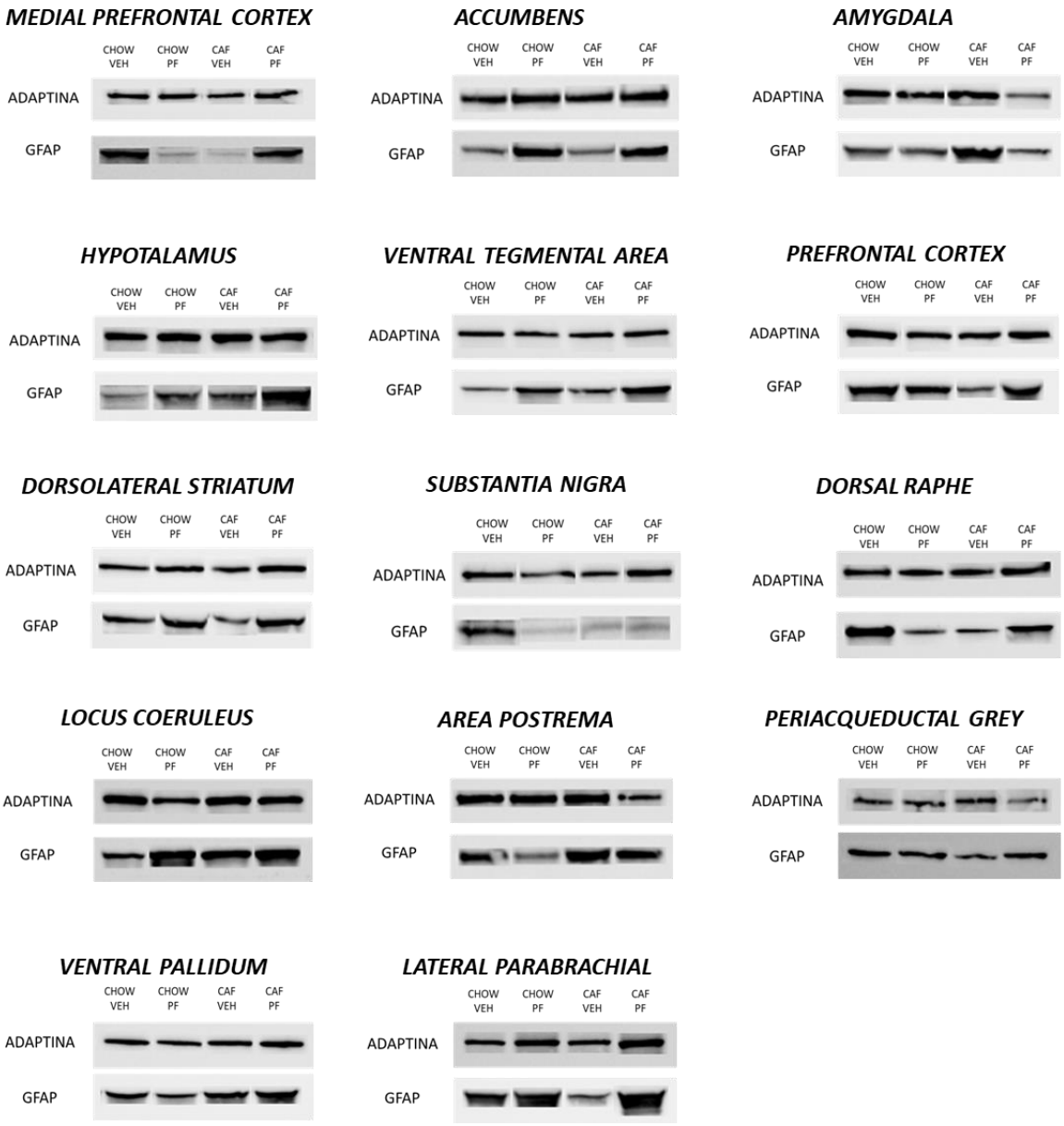

Fig.50: Representative images of western blot analysis of GFAP.

On the other hand, expression of IBA-1 was significantly affected in AMY: animals abstinent from the diet (CAF VEH) displayed a significant reduction of the expression of the protein compared to CHOW-fed ones (CHOW VEH). The administration of PF-3845 was effective in causing a significant increase of the expression of IBA-1 in AMY, SN and DR in CAFETERIA abstinent rats (CAF PF), if compared to CAF VEH group. Administration of PF-3845 in chow-fed animals (CHOW PF) significantly reduced IBA-1 expression in LC when compared to CHOW VEH. When the same compound was administered in CAF PF animals, we reported a significant increase in the expression of IBA-1 in DR and LC when compared to CHOW PF group.

Results obtained from two-way ANOVA analyses conducted for each parameter are reported in Table 17, whereas the results from the *post hoc* analyses (Tukey's test) are reported in figure 51. Figure 52 shows representative images of western blot analysis.

Two-way ANOVA for IBA-1 expression in the different areas

	F diet		F treatment		F interaction		df
PFC	0,910	(p=0,350)	0,947	(p=0,341)	1,305	(p=0,266)	23
mPFC	0,532	(p=0,473)	0,802	(p=0,380)	0,017	(p=0,897)	23
ACC	0,013	(p=0,910)	0,048	(p=0,829)	**4,673**	***(p<0,05)***	22
DLS	0,702	(p=0,410)	0,414	(p=0,526)	1,162	(p=0,292)	24
AMY	0,258	(p=0,617)	1,646	(p=0,213)	**9,930**	***(p<0,01)***	23
HYPO	0,171	(p=0,683)	0,022	(p=0,883)	2,513	(p=0,127)	23
VPL	1,100	(p=0,305)	0,967	(p=0,335)	0,236	(p=0,632)	25
DR	0,675	(p=0,419)	3,722	(p=0,066)	**9,383**	***(p<0,01)***	25
VTA	1,470	(p=0,237)	3,738	(p=0,065)	0,298	(p=0,590)	23
SN	0,614	(p=0,442)	1,704	(p=0,205)	2,971	(p=0,099)	23
LC	**9,581**	***(p<0,01)***	1,178	(p=0,290)	4,080	(p=0,055)	23
LPB	0,367	(p=0,550)	0,059	(p=0,810)	0,440	(p=0,514)	24

Table 17: Two-way ANOVA analysis results for the expression of IBA-1 in various areas. The two factors considered were diet (CHOW, CAF) and treatment (VEH, PF). Diet was a significant factor in LC. A significant interaction between the two factors was detected in ACC, AMY and DR. All significant values are written in italics in the table.

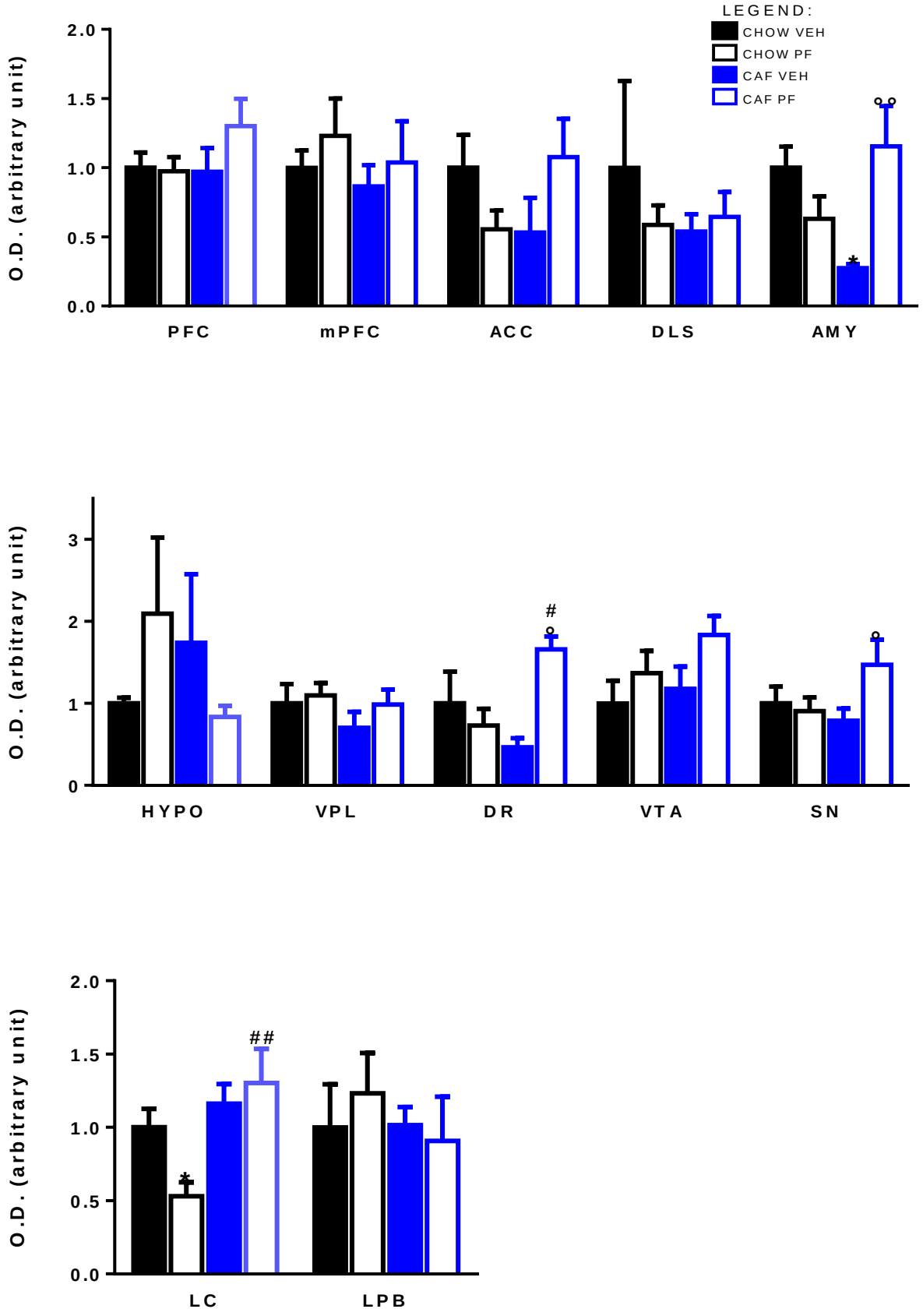

Fig.51: Results of the western blot analysis of IBA-1 expression; expressed as the protein/adaptin ratio. Results are expressed as MEAN±SEM. *p<0,05 vs CHOW VEH; °p<0,05 vs CAF VEH; °°p<0,01 vs CAF VEH; $^{\#}$p<0,05 vs CHOW PF; $^{\#\#}$p<0,01 vs CHOW PF (Tukey's post-hoc test for between-groups comparisons).

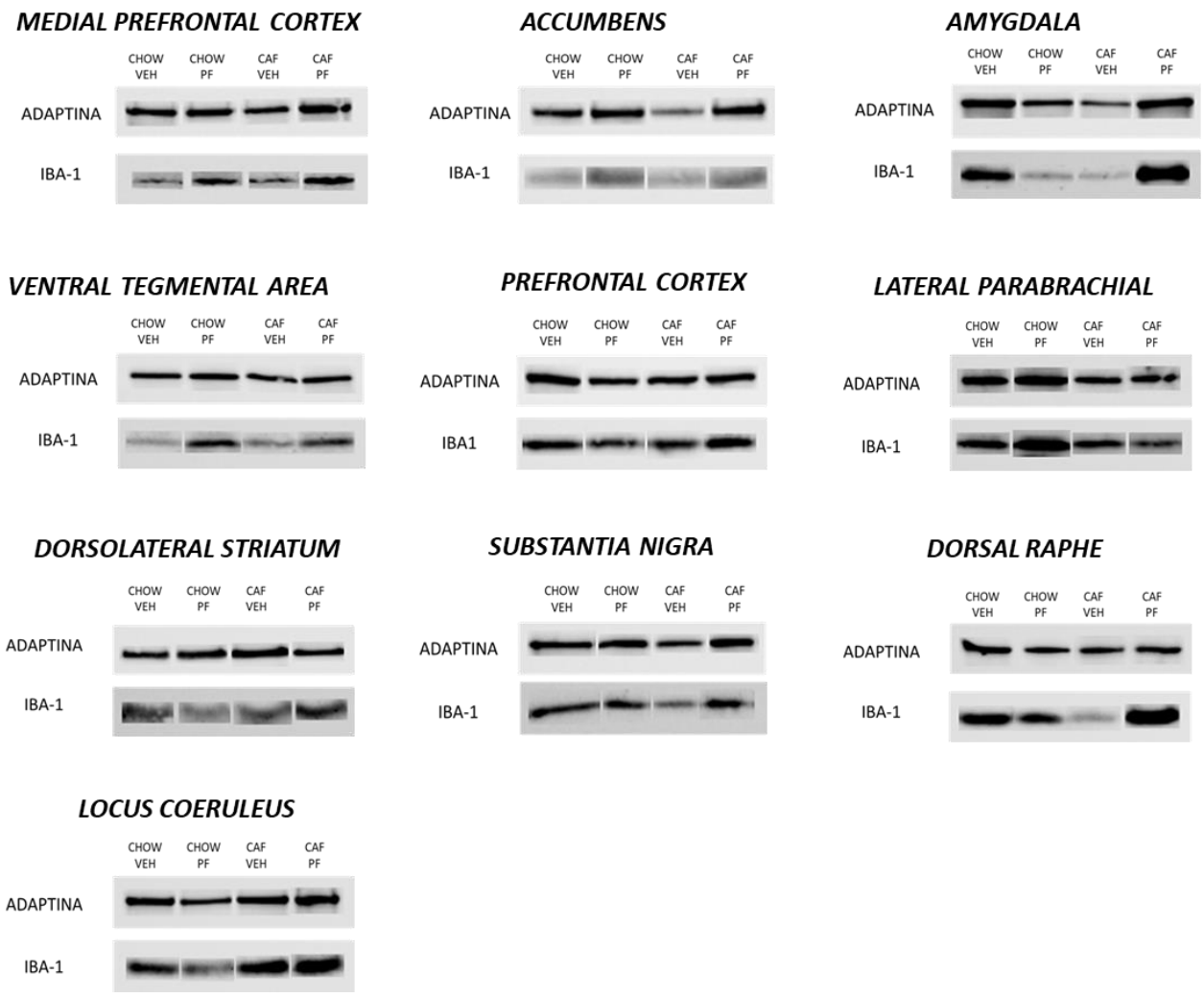

Fig.52: Representative images of western blot analysis of IBA-1.

COX-2 expression analysis in HYPO revealed that PF-3845 administration in animals abstinent from the diet (CAF PF group) was effective in reducing COX-2 significantly when compared to vehicle-treated animals (CAF VEH group). Two-way ANOVA analysis revealed that nor diet *(F=0,420; p=0,524; df=23)* neither treatment *(F=2,604; p=0,120; df=23)* were significant factors. No interaction between the two factors was displayed *(F=1,830; p= 0,193; df=23)*. Tukey's post-hoc analysis was performed; results are shown in figure 53. Representative image of western blot analysis is shown in figure 54.

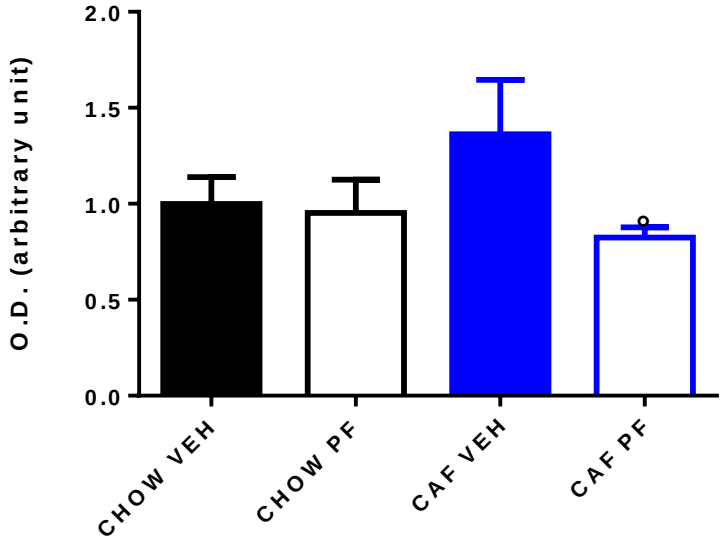

Fig.53: Results of the western blot analysis of COX-2 expression; expressed as the protein/adaptin ratio. Results are expressed as MEAN±SEM. °p<0,05 vs CAF VEH (Tukey's post-hoc test for between-groups comparisons).

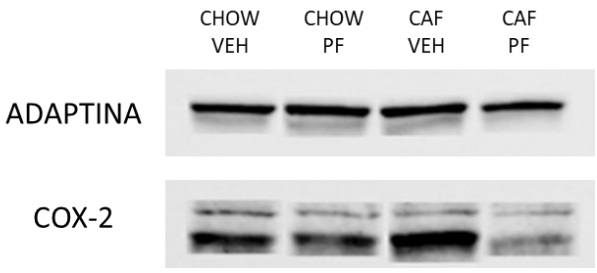

Fig.52: Representative images of western blot analysis of COX-2.

6. PEARSON CORRELATION ANALYSIS:

A Pearson correlation is a number between -1 and 1 that indicates the extent to which two variables are linearly related. Correlations are never lower than -1. A correlation of -1 indicates that the data points in a scatter plot lie exactly on a straight descending line; the two variables are perfectly negatively linearly related; whereas a correlation of 0 means that two variables do not have any linear relation whatsoever. At the same time, correlation coefficients are never higher than 1. A correlation coefficient of 1 means that two variables are perfectly positively linearly related; the dots in a scatter plot lie exactly on a straight ascending line.

In our study, we performed various Pearson correlation analyses, considering matching data per each animal included in the results:

- Correlative analysis between selected parameters of behavioural tests and monoamines concentration among areas: the aim is to discover if the variation in the concentration of monoamines in selected brain regions could be directly related to behavioural alterations and if the exposure and subsequent abstinence from CAFETERIA diet or pharmacological treatment might have an impact on these correlations;
- Correlative analysis of monoamines across different brain regions: the aim is to understand which regions are interconnected in DA, NA, 5HT systems and if the exposure and subsequent abstinence from CAFETERIA diet or pharmacological treatment might have an impact on these correlations;
- Correlative analysis of proteins partaking to the endocannabinoid system across different brain regions: the aim is to discover if the different proteins interact in different areas and if the exposure and subsequent abstinence from CAFETERIA diet or pharmacological treatment might have an impact on these correlations;
- Correlative analysis of monoamine and cannabinoid system components in the same brain region: the aim is to discover how, in every brain region that we analysed, the endocannabinoid system and the monoaminergic one are related to each other and if the exposure and subsequent abstinence from CAFETERIA diet or pharmacological treatment might have an impact on these correlations.

6.1 CORRELATIVE ANALYSIS OF BEHAVIOURAL PARAMETERS IN RELATION TO MONOAMINE CONCENTRATION:

To test the existence of a selective relationship between behavioural alterations and monoamine tissue levels across the different brain regions we measured the coefficient of correlation between selected behavioural parameters and concentration of the monoamines in each area. Pearson correlation analysis was executed for each monoamine separately, and the results for each experimental group were compared.

The parameters that were chosen as indicative of each behavioural test were:

- Zone entries for the OFT
- Time spent in open arms and centre for EPM
- Mobility time for FST

DOPAMINE:

In figure 53 it is possible to observe the correlation pattern between behavioural tests parameters and DA content in the different brain areas. Interestingly, the number of correlations between the different groups did not vary a lot; but at the same time diet abstinence and pharmacological treatment can modify the pattern of correlation. For example, CAF VEH animals presented a positive correlation between zone entries in OF and mobility time in FST and a negative one between concentration of DA in mPFC and zone entries; differently from CHOW VEH group, which refers to a "normal" condition, where the only correlation seen is between mobility time of FST and DA concentration in dHIPPO. Administration of PF-3845 in rats abstinent from cafeteria diet created a pattern different from both CAF VEH and CHOW VEH groups: it was possible to observe a negative correlation between DA concentration in PFC and zone entries.

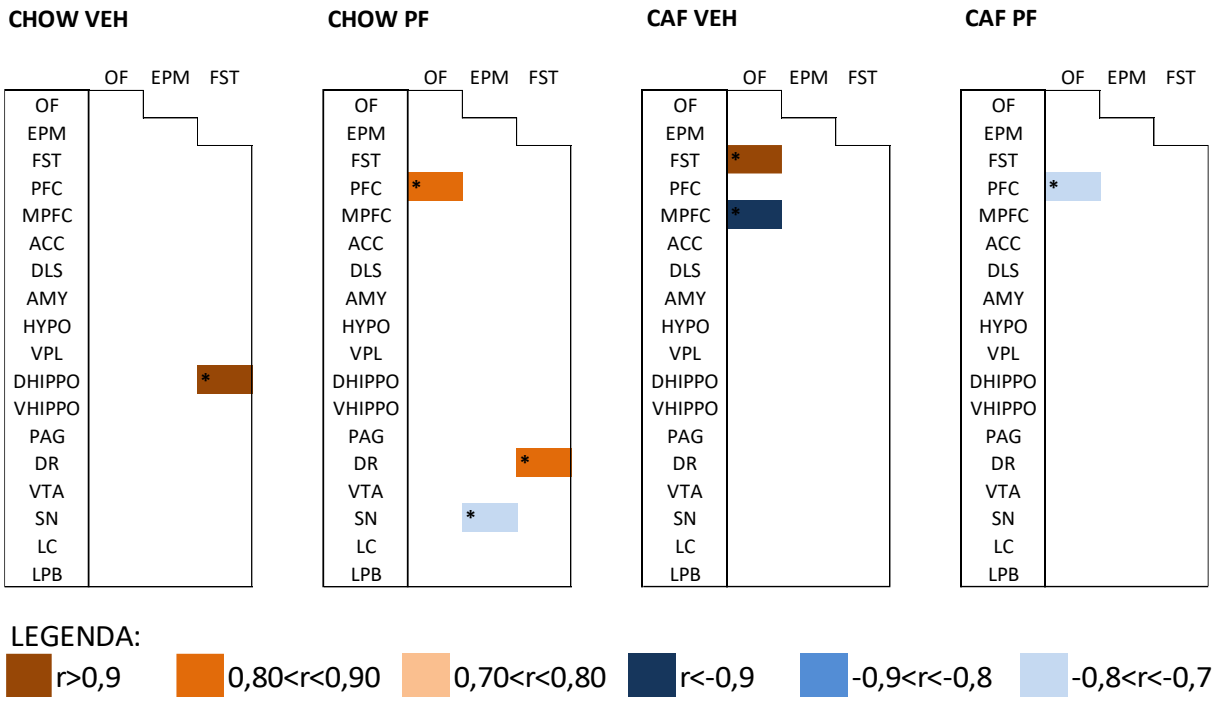

Fig.53: Pearson's correlation analysis results for behavioural parameters and DA concentration in the various brain areas. Only significant correlations are displayed (*p<0,05).

NORADRENALINE:

In figure 54 it is possible to observe the correlation pattern between behavioural tests and NA content in the different brain areas. Likewise to what happened for DA, the correlations between NA tissue content and behavioural parameters did not increase or decrease in number due to diet abstinence or pharmacological treatment. What is possible to observe is that CHOW VEH animals presented a correlation between time spent in centre and open arms and the concentration of NA in dHIPPO; a correlation that is loss due to diet abstinence in CAF VEH group. Administration of PF-3845 in animals abstinent from cafeteria diet was not able to re-establish a "normal" pattern; but was linked to the presence of a correlation between zone entries and NA content in ACC (negative) and in VTA (positive).

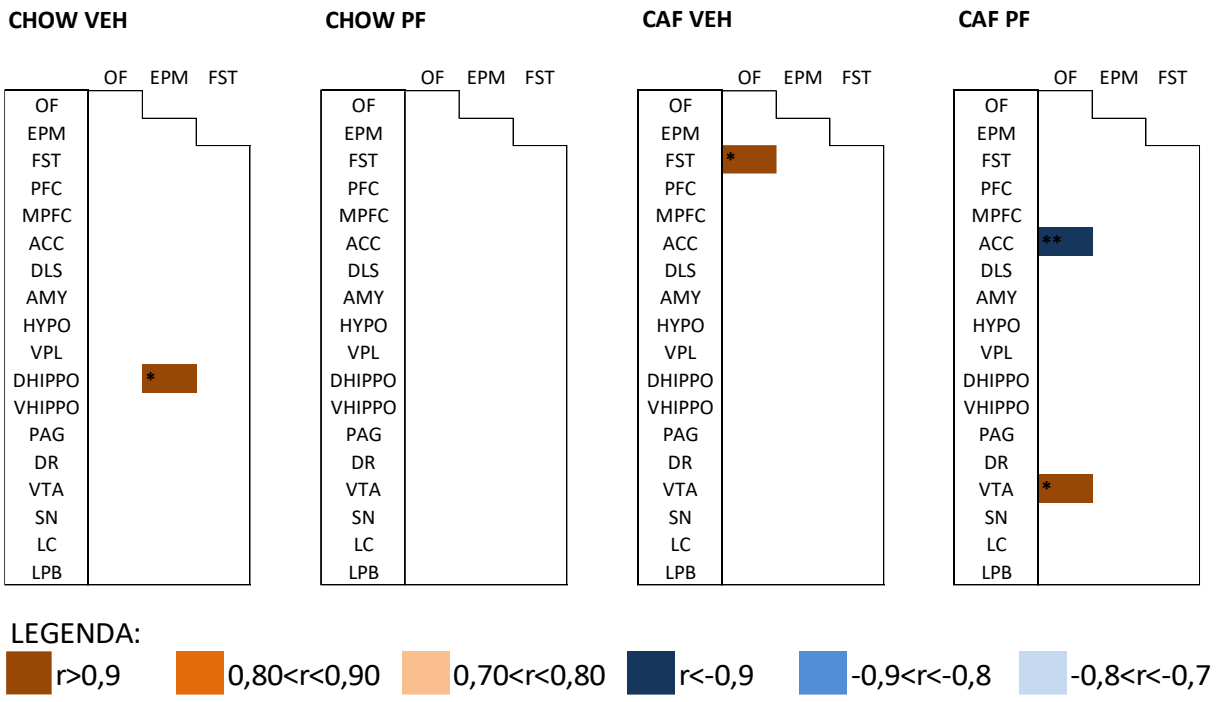

Fig.54: Pearson's correlation analysis results for behavioural parameters and NA concentration in the various brain areas. Only significant correlations are displayed (*p<0,05).

SEROTONIN:

In figure 55 it is possible to observe the correlation pattern between behavioural tests and 5HT content in the different brain areas. Differently from DA and NA, the number of correlations between 5HT and behavioural test parameters varied due to diet abstinence and pharmacological treatment. In particular, CAF VEH rats after experiencing diet abstinence presented an increase in the number of correlations: zone entries correlate negatively with serotonin concentrations in mPFC, VPL, vHIPPO; whereas mobility time correlates positively with 5HT tissue content in VTA. This pattern resulted strongly different to the one corresponding to CHOW VEH group, used as a reference for a "normal" condition, where 5HT concentration in dHIPPO correlated with time spent in centre and open arms during EPM. In this case, the pharmacological treatment in CAF PF animals was able to create a pattern similar to CHOW VEH, considering that no significative correlations are reported.

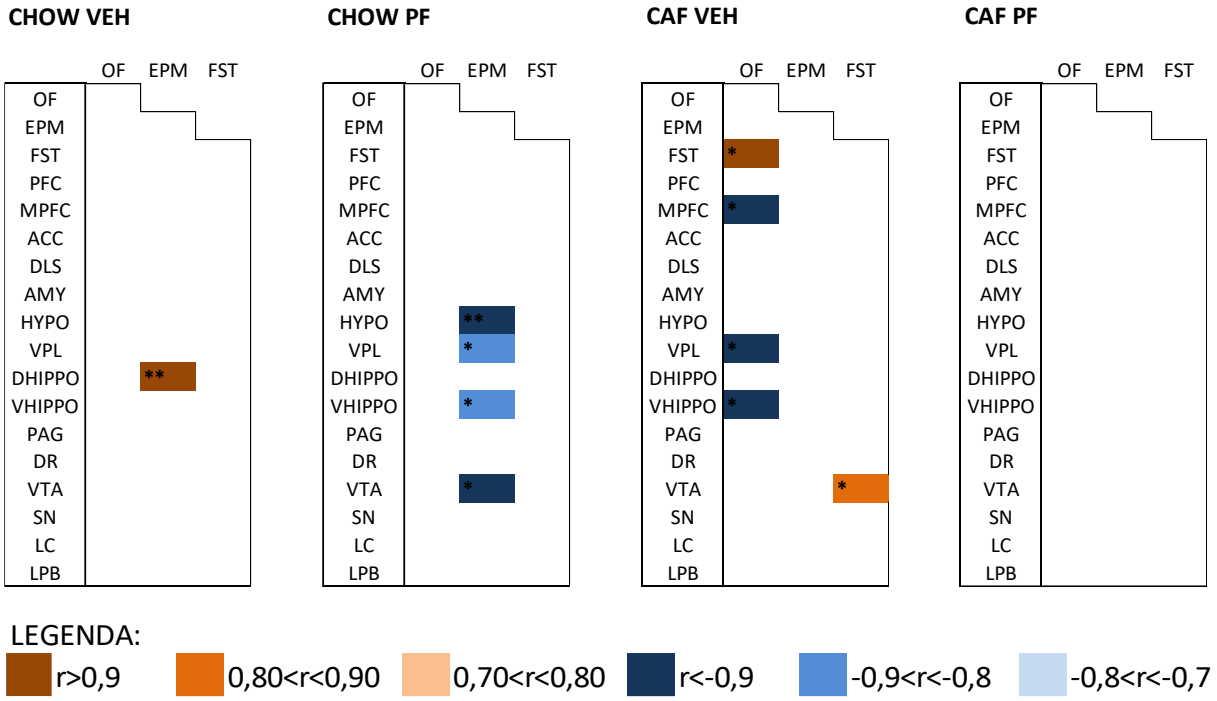

Fig.55: Pearson's correlation analysis results for behavioural parameters and 5HT concentration in the various brain areas. Only significant correlations are displayed (*p<0,05; **p<0,01).

DOPAMINE TURNOVER:

In figure 56 it is possible to observe the correlation pattern between behavioural tests and DA turnover in the different brain areas. Likewise, to what happen for DA, cafeteria abstinence did not vary the number of correlations between CHOW VEH and CAF VEH group, but varies the parameters which correlate to each other. In CHOW VEH animals, mobility time correlated with DA turnover both in PFC and DLS positively. Differently, in CAF VEH animals VTA's DA turnover correlated negatively with mobility time. PF-3845 administration varied correlations in CAF PF animals, in a pattern different both to CAF VEH and CHOW VEH animals; where DA turnover in ACC relates negatively with mobility time of FST.

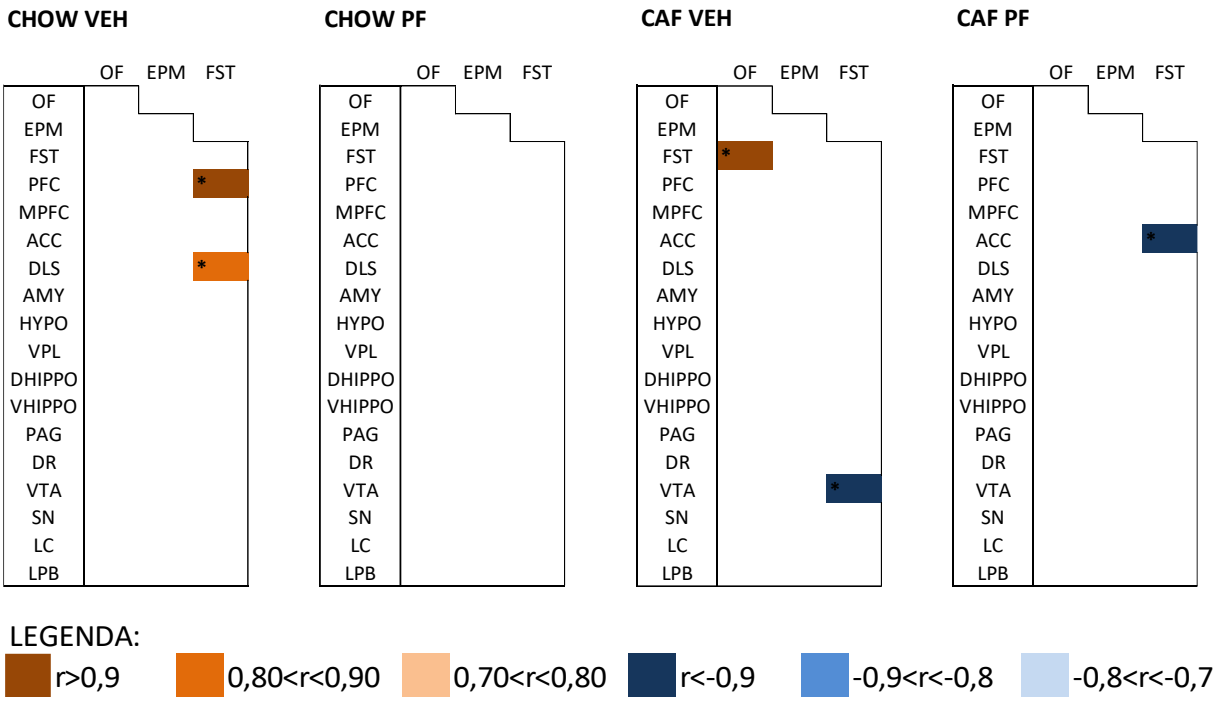

Fig.56: Pearson's correlation analysis results for behavioural parameters and DA turnover in the various brain areas. Only significant correlations are displayed (*p<0,05).

SEROTONIN TURNOVER:

In figure 57 it is possible to observe the correlation pattern between behavioural tests and 5HT turnover in the different brain areas. Similarly, to the patterns observed for 5HT, the number of correlations in CAF VEH animals is higher than CHOW VEH. In particular, zone entries correlated positively with 5HT turnover in PFC, HYPO and negatively with the turnover in vHIPPO and VTA. At the same time, mobility time correlated positively with 5HT turnover in HYPO and negatively with ACC and VTA. This pattern appeared strongly different to CHOW VEH one, where 5HT turnover in SN correlates with zone entries and in AMY with time spent in centre and open arms. Pharmacological treatment was able to reduce the number of correlations in CAF PF animals, in fact the only correlations shown were between 5HT turnover in HYPO and zone entries, turnover in the AMY and time spent in centre and open arms and turnover in VTA and mobility time.

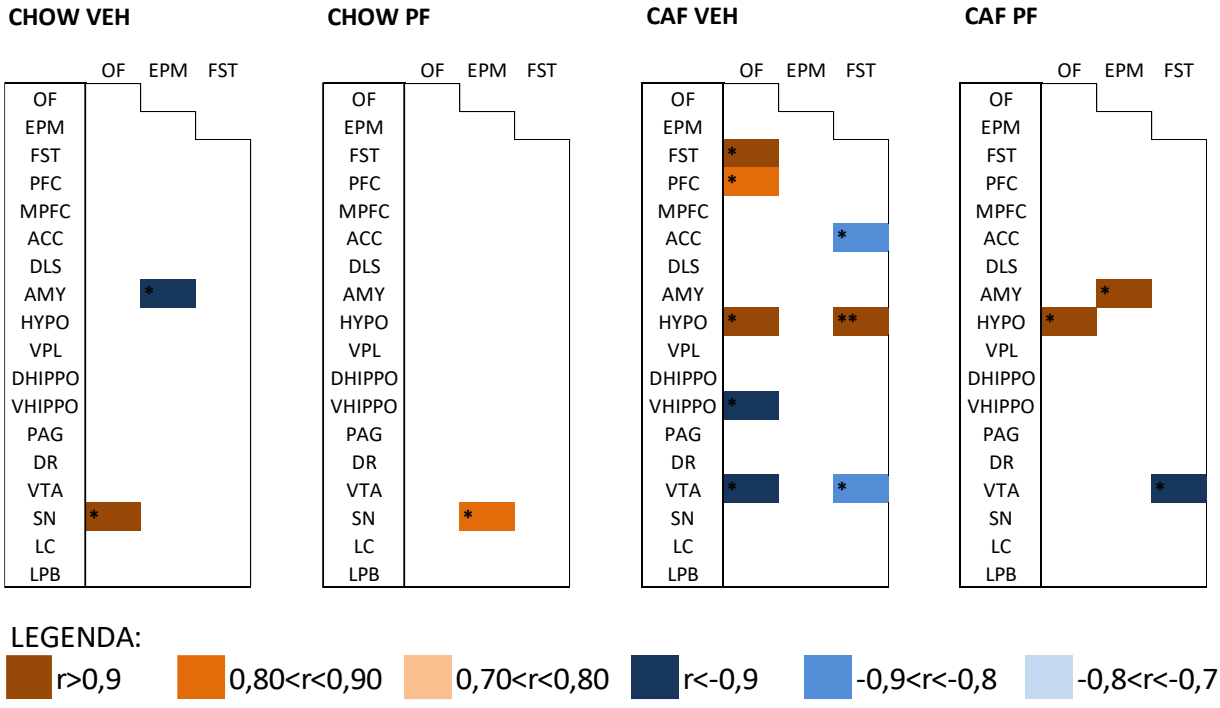

Fig.57: Pearson's correlation analysis results for behavioural parameters and 5HT turnover in the various brain areas. Only significant correlations are displayed (*p<0,05; **p<0,01).

6.2 CORRELATIVE ANALYSIS OF MONOAMINES ACROSS DIFFERENT BRAIN REGIONS:

To test the presence of a selective relationship between monoamine levels across the different brain regions we measured the coefficient of correlation between the monoamine content in paired brain structures. Pearson correlation analysis was performed for each monoamine separately, and the results for each experimental group were compared.

DOPAMINE:

DA patterns of correlation varied across the different groups: it's interesting to notice how CAF VEH animals, after experiencing diet abstinence, presented the same number of correlations (7 out of 120) of CHOW VEH group (which represents a "normal" condition). However, the pattern of correlation appeared strongly different: the two groups have in common only a positive correlation between DA concentration in mPFC and PFC; suggesting that diet abstinence can vary the areas that use DA to communicate to each other. PF-3845 administration in animals experiencing diet abstinence (CAF PF) was not able to restore a normal condition: it was possible to observe how there is a little increment of correlations (9 out of 120), but once again the areas that correlated to each other generated a pattern which is different both from CAF VEH and CHOW VEH ones. Figure 58 shows the in-between area correlations for each experimental group, r and p values for each significant correlation.

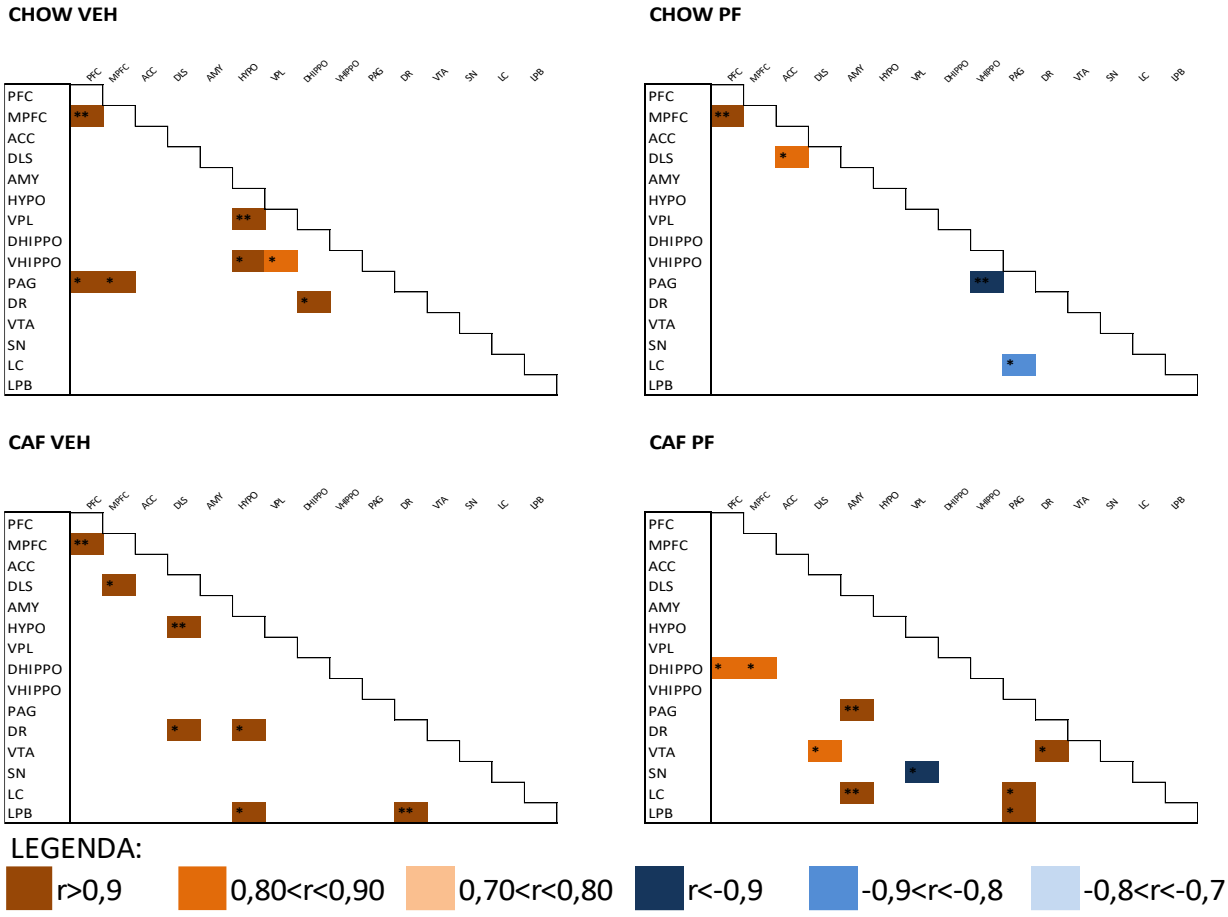

Fig.58: Pearson's correlation analysis results for DA. Only significant correlations are displayed (*p<0,05; **p<0,01).

NORADRENALINE:

Pearson correlation analysis for NA evidenced both in CHOW VEH and in CAF VEH animals a low grade of in-between correlations (9 out of 120 for CHOW VEH and 8 out of 120 for CAF VEH). However, it is important to notice that CAF VEH animals displayed a correlation pattern completely different from CHOW VEH, suggesting that abstinence from CAFETERIA diet might vary the interconnections between the different areas. PF-3845 treatment, in this case, was able to increase the number of correlations in CHOW PF animals (14 out of 120) but not in CAF PF animals (6 out of 120). In both cases, the pattern resulting from PF-3845 administration was not superimposable to the one of a "normal" condition. Figure 59 shows the in-between area correlations for each experimental group, r and p values for each significant correlation.

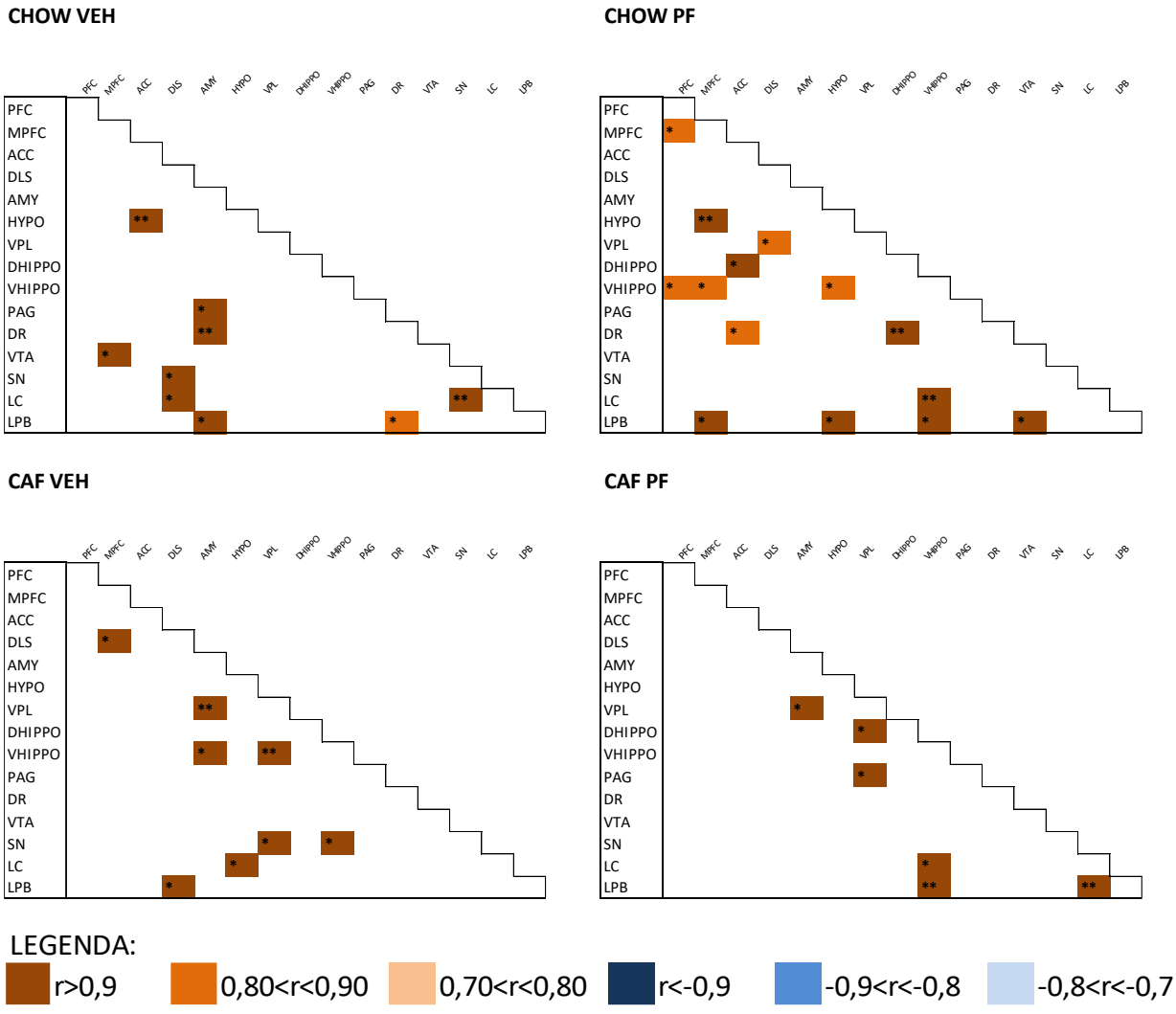

Fig.59: Pearson's correlation analysis results for NA. Only significant correlations are displayed (*p<0,05; **p<0,01).

SEROTONIN:

Pearson correlation analysis for 5HT displayed a high rate of in-between area correlations. In particular, CHOW VEH group presented 16 correlations between areas out of 120. Diet abstinence, in CAF VEH group, was able not only to decrease the number of these correlations (11 out of 120) but at the same time also to vary the areas which correlate; as shown in figure 60. We can justify this result imaging that diet abstinence, in this case, can decrease serotonergic transmission and at the same time modify the areas that use the neurotransmitter to communicate with each other. PF-3845 treatment, in CAF PF group, was able to increase significantly the number (27 out of 120) and normalize the pattern of correlations; it was possible to see how CAF PF pattern appears more superimposable to CHOW VEH one when compared to CAF VEH; thus suggesting that pharmacological treatment can "normalize" the serotonergic transmission between the different areas of the brain. Figure 60 shows the in-between area correlations for each experimental group, r and p values for each significant correlation.

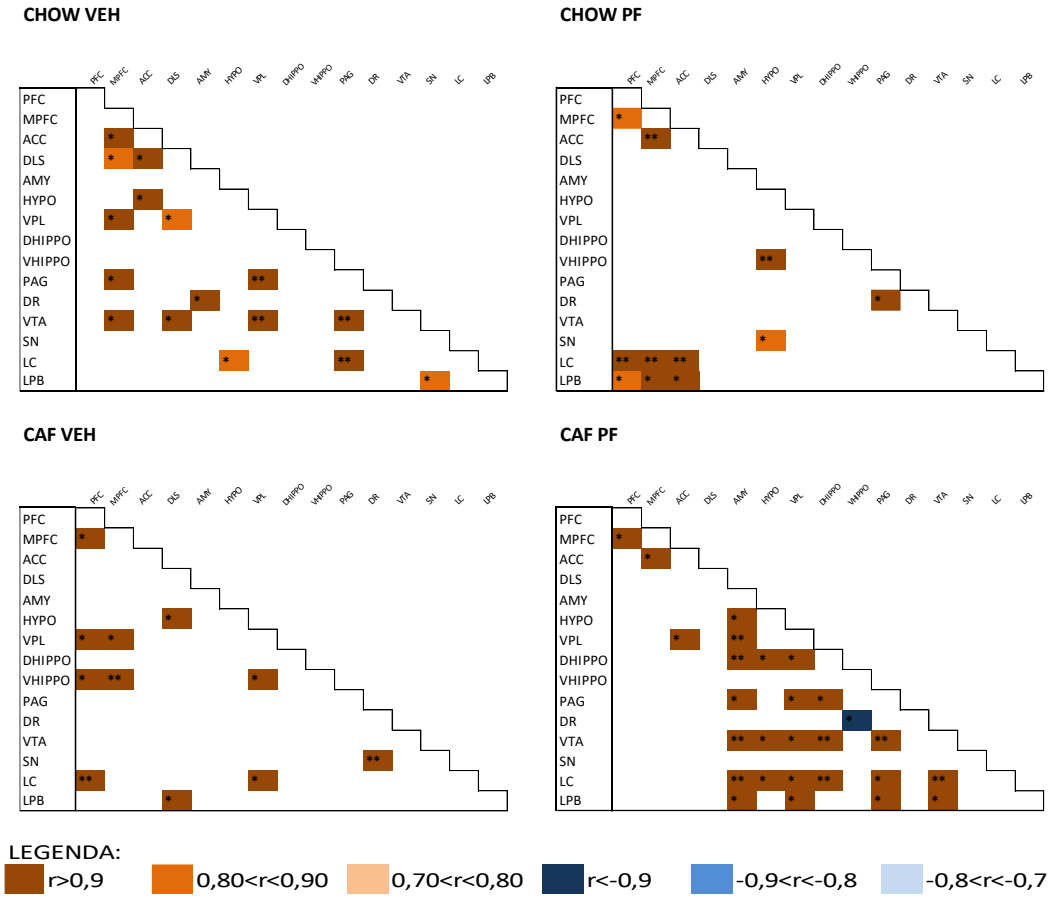

Fig.60: Pearson's correlation analysis results for 5HT. Only significant correlations are displayed (*p<0,05; **p<0,01).

DOPAMINE TURNOVER:

Pearson correlation analysis for DA turnover evidenced both in CHOW VEH and in CAF VEH animals a low grade of in-between correlations (4 out of 120). Even if the number of correlations was the same between the two groups, the pattern varies, thus suggesting that diet abstinence can affect not only the DA neurotransmitter per se (like has been shown before), but also its turnover. PF-3845 administration was able to increase the number of correlations both in CHOW PF and CAF PF treated animals, suggesting that the drug is acting by stimulating the dopaminergic system.

Figure 61 shows the in-between area correlations for each experimental group, r and p values for each significant correlation of DA turnover.

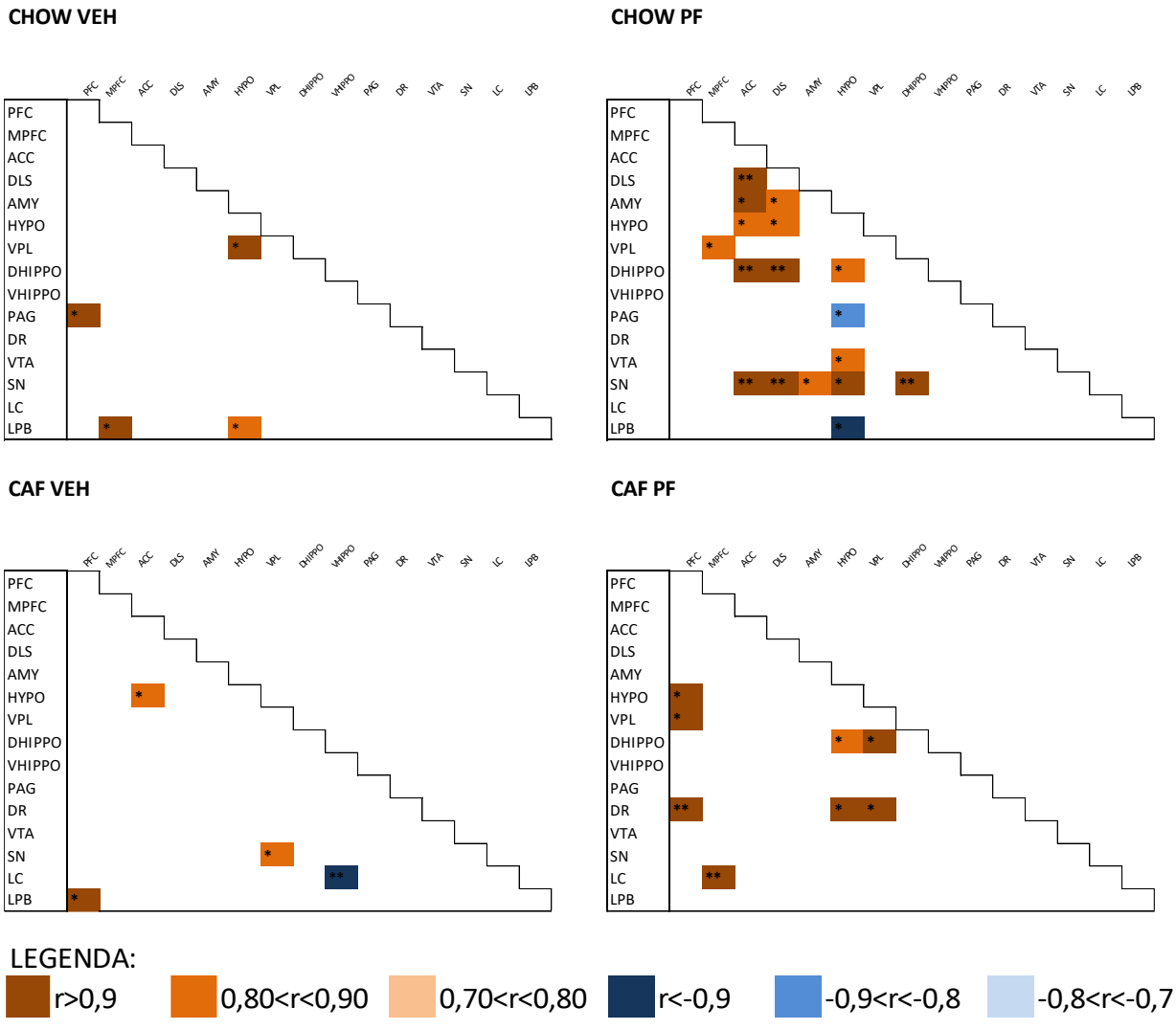

Fig.61: Pearson's correlation analysis results for DA turnover. Only significant correlations are displayed (*p<0,05; **p<0,01).

SEROTONIN TURNOVER:

Differently from what has been observed for serotonin, the study of the Pearson correlation of 5HT turnover among the different areas revealed a low grade of in-between area correlations in CHOW VEH, CAF VEH and CAF PF groups. Also in this case, even if the number of correlations did not vary between the different groups, there was a variation of the correlation pattern due to diet abstinence and pharmacological treatment.

Figure 62 shows the in-between area correlations for each experimental group, r and p values for each significant correlation of DA turnover.

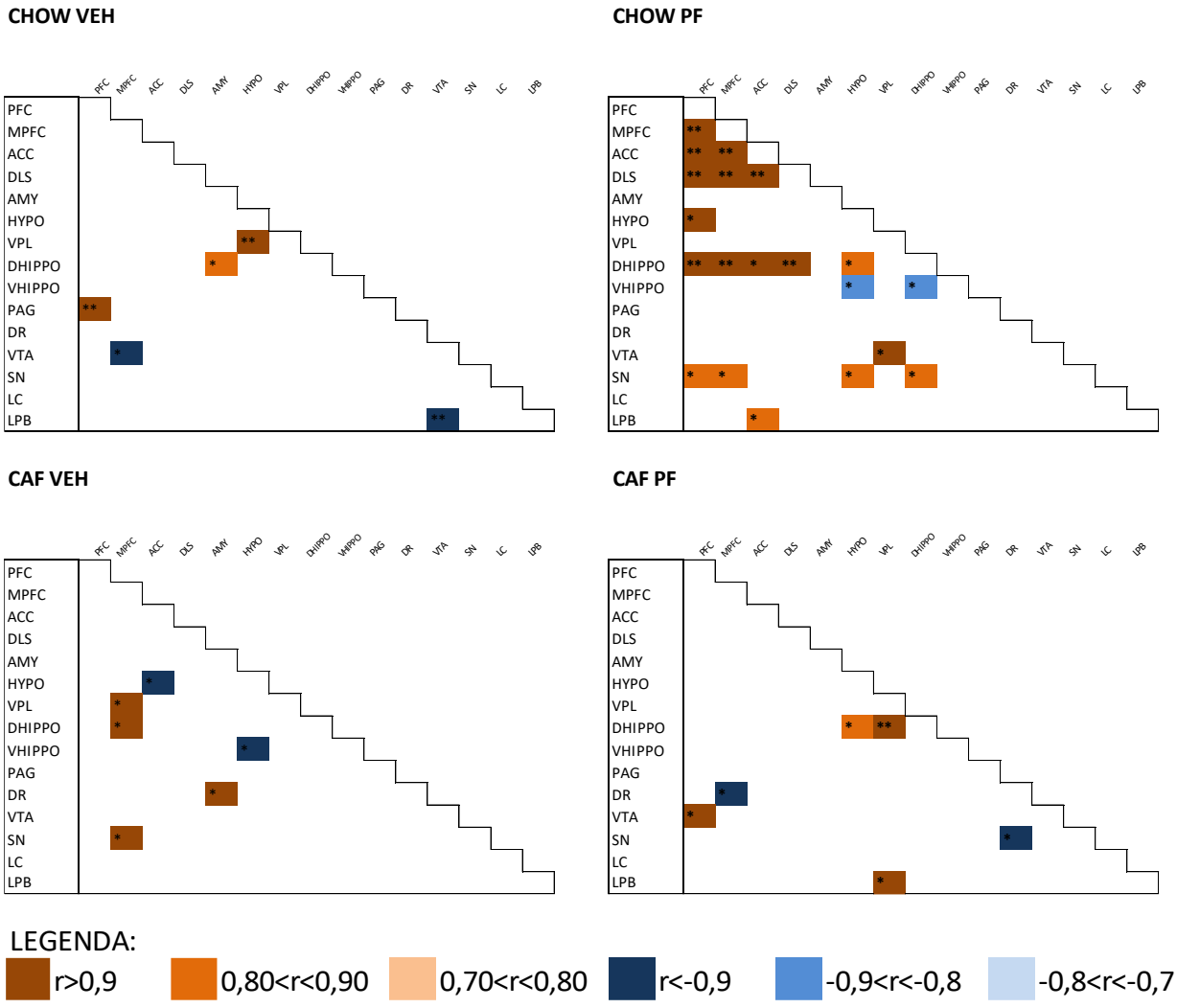

Fig.62: Pearson's correlation analysis results for 5HT turnover. Only significant correlations are displayed (*p<0,05; **p<0,01).

6.3 CORRELATIVE ANALYSIS OF CANNABINOID SYSTEM COMPONENTS AND INFLAMMATORY PROTEINS ACROSS DIFFERENT BRAIN REGIONS:

To test the existence of a selective relationship between the levels of the proteins partaking to the endocannabinoid system across the different brain regions, we measured the coefficient of correlation between the protein content in paired brain structures. Pearson correlation analysis was performed for each protein separately, and the results obtained from each experimental group were compared.

CB1:

Pearson correlation analysis for CB1 expression in the different brain areas revealed that diet abstinence was able to vary the pattern of correlation in CAF VEH animals when compared to the "normal" condition, that is CHOW VEH group. In fact, there was a variation both of the areas which are functionally linked and of the type of correlation (mostly negative in CHOW VEH and positive in CAF VEH). Administration of PF-3845 in CAF PF animals was not able in this case to "normalize" the pattern, even though it increases the number of the correlations.

Figure 63 shows the pattern of correlation for CB1 receptor in the various brain areas.

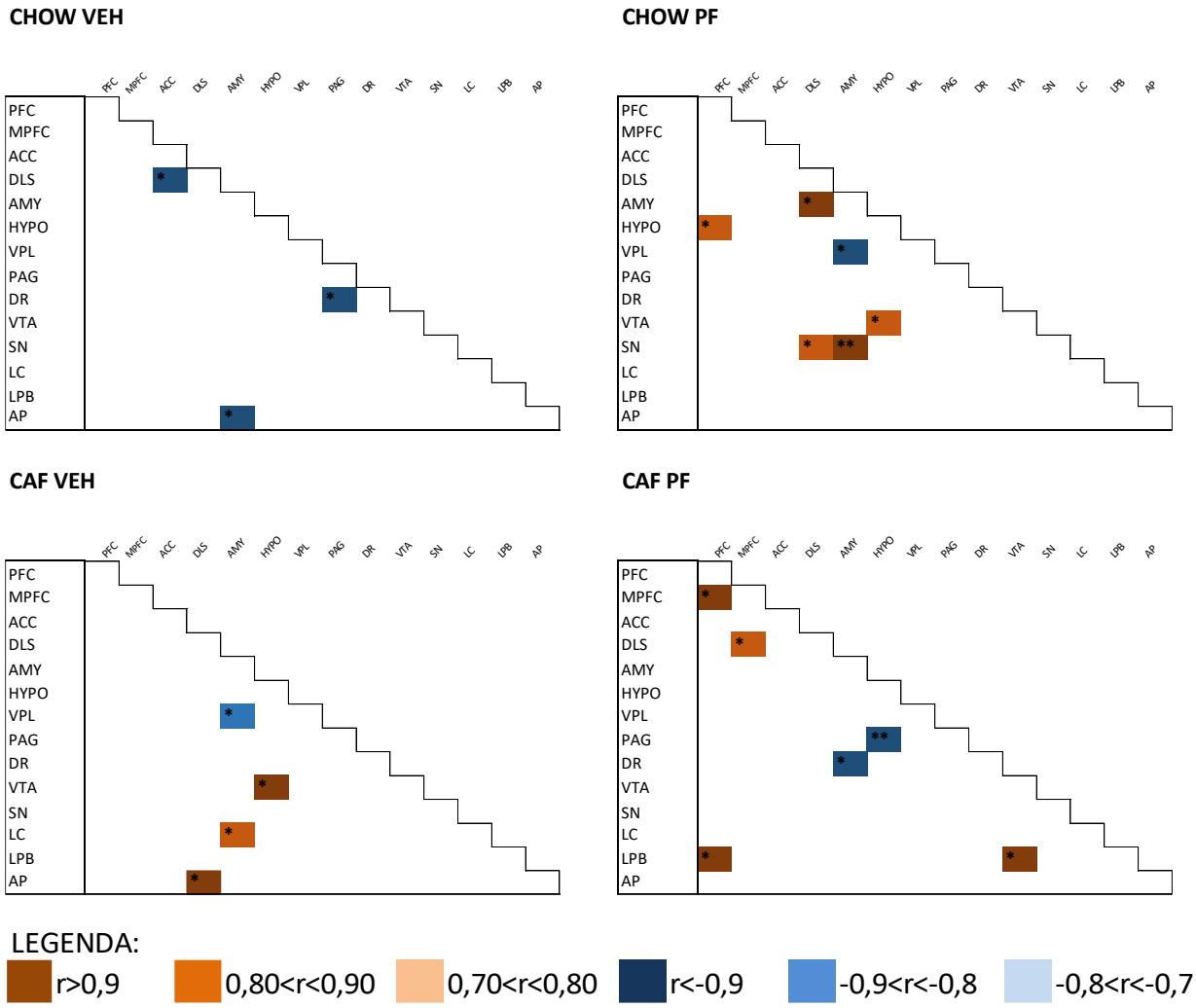

Fig.63: Pearson's correlation analysis results for CB1 receptor. Only significant correlations are displayed (*p<0,05; **p<0,01).

CB2:

Differently from CB1, Pearson correlation analysis for CB2 expression revealed a low number of in-between areas correlations. However, also in this case CAF VEH animals displayed a different pattern when compared to CHOW VEH ones; and pharmacological treatment in CAF PF group did not seem effective in normalizing it.

Figure 64 shows the pattern of correlation for CB2 receptor in the different brain areas analysed.

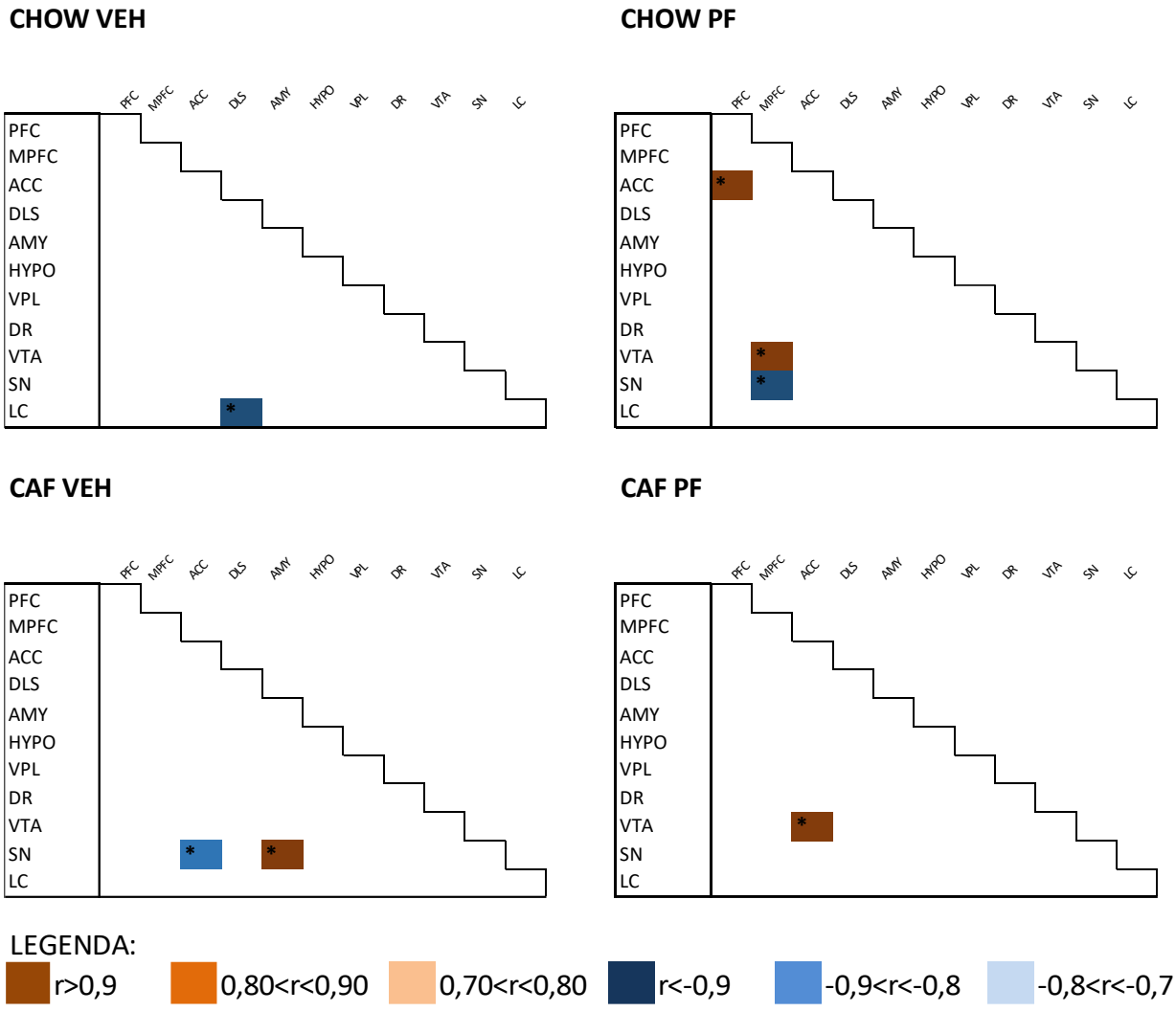

Fig.64: Pearson's correlation analysis results for CB2 receptor. Only significant correlations are displayed (*p<0,05; **p<0,01).

FAAH:

Pearson correlation analysis for the expression of FAAH in the various brain areas revealed the same number of correlations in both CAF VEH and CHOW VEH groups. However, the correlation pattern between the two groups was completely different, suggesting that diet abstinence was able to vary the functional connection between the different brain areas. Once more, PF-3845 treatment was not able to re-establish a normal correlation pattern in CAF PF group.

Figure 65 shows the pattern of correlation for FAAH in the different brain areas analysed.

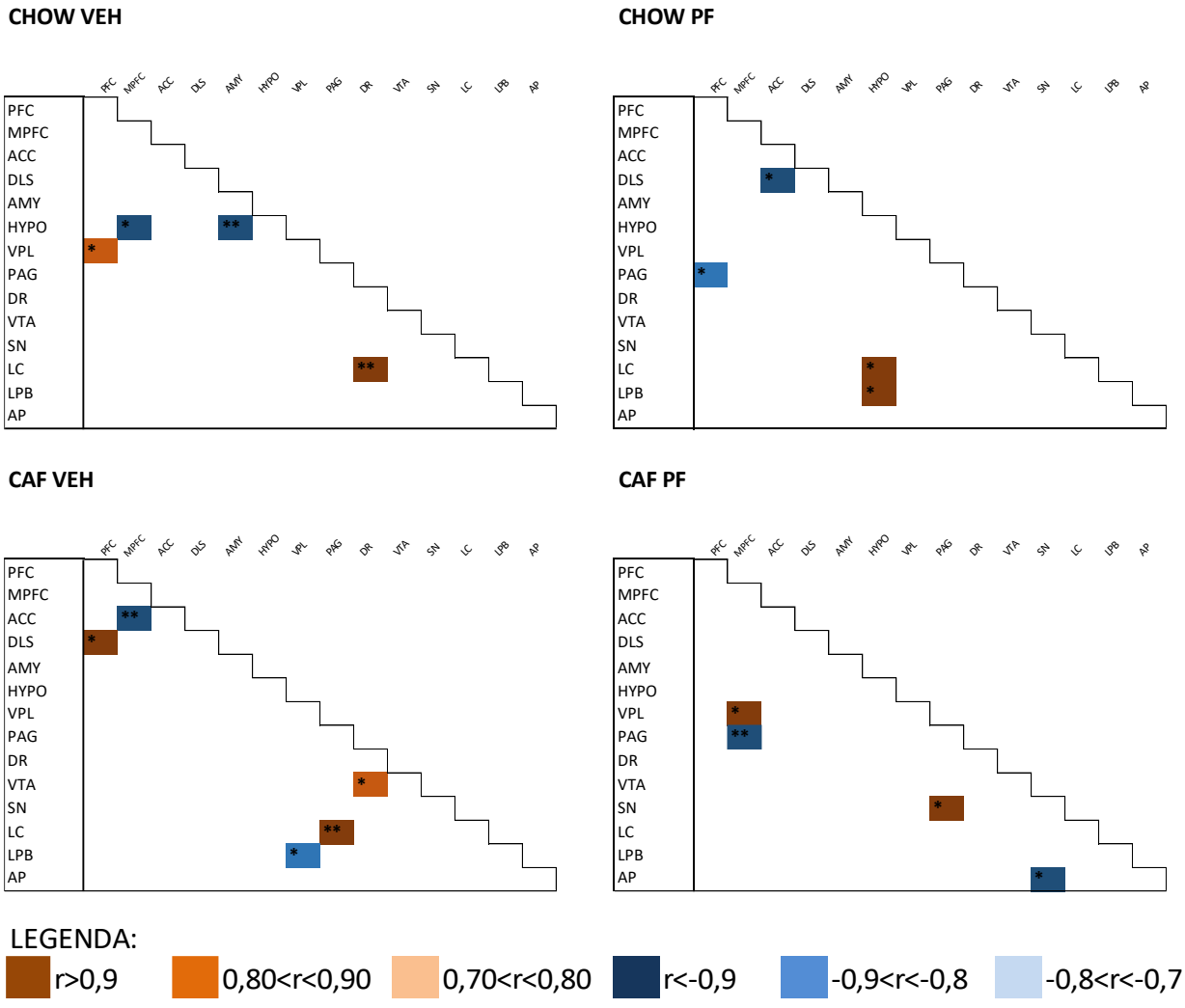

Fig.65: Pearson's correlation analysis results for FAAH. Only significant correlations are displayed (*p<0,05; **p<0,01).

MAGL:

Correlation analysis of MAGL protein showed an increment of the number of correlations in CAF VEH animals; suggesting that diet abstinence was able to influence the activity of this protein in different brain regions. Moreover, differently from CHOW VEH group, CAF VEH animals displayed a large number of positive correlations. In this case, PF-3845 administration in animals abstinent from the diet was effective in normalizing the correlation pattern: CAF PF group showed no significant correlation, so pharmacological treatment was effective in reducing the number of correlations.

Figure 66 shows the pattern of correlation for MAGL in the different brain areas analysed.

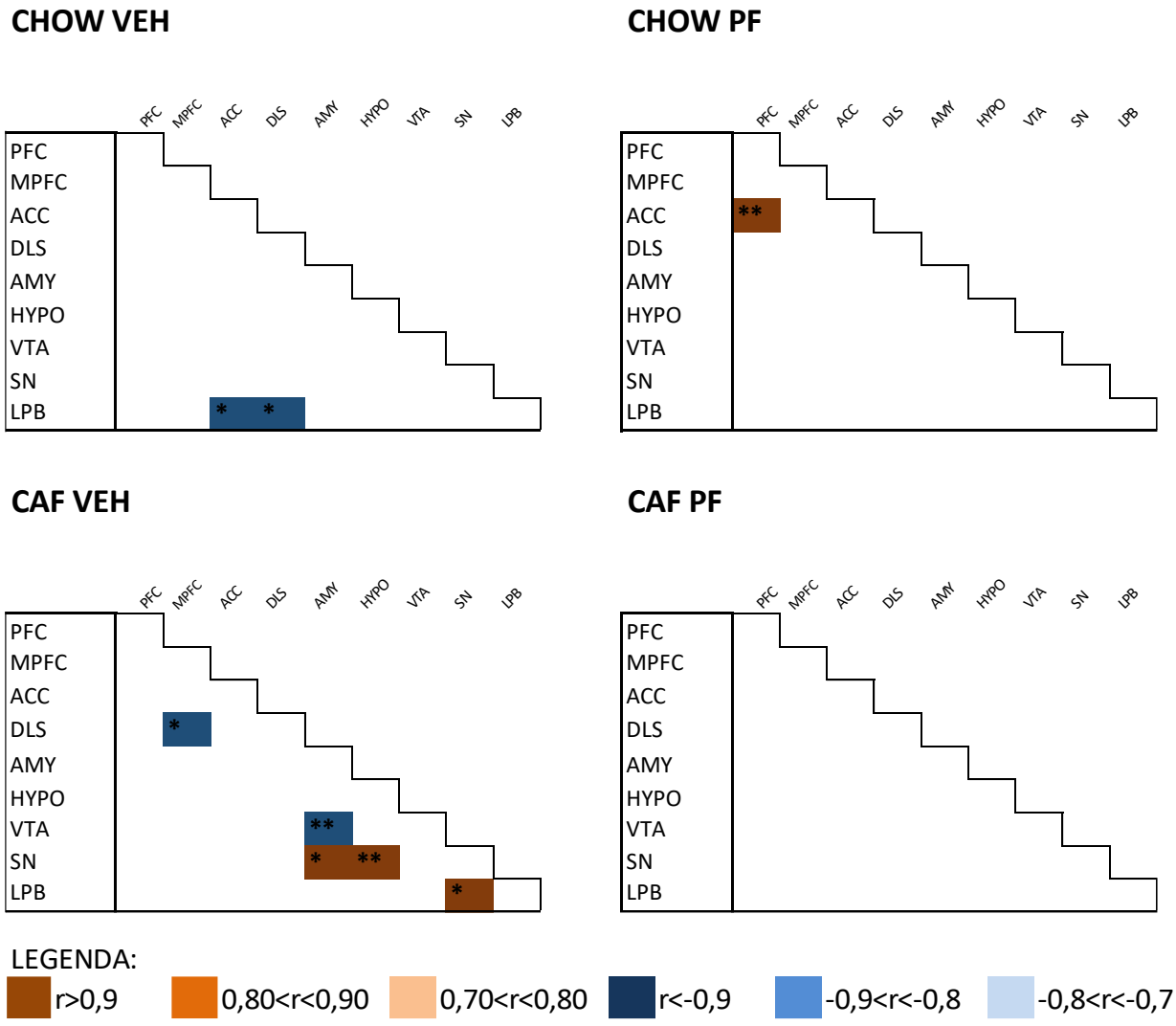

Fig.66: Pearson's correlation analysis results for MAGL. Only significant correlations are displayed (*p<0,05; **p<0,01).

NAPE PLD:

Diet abstinence is able to affect also the pattern of correlation of NAPE-PLD: CAF VEH animals displayed an increment of the correlations when compared to CHOW VEH ones, thus suggesting once again that the functional relations between the areas could be compromised by diet withdrawal. Pharmacological treatment in CAF PF animals was able to restore a pattern of correlation completely superimposable to CHOW VEH, thus sustaining its effectiveness.

Figure 67 shows the pattern of correlation for NAPE-PLD in the different brain areas analysed.

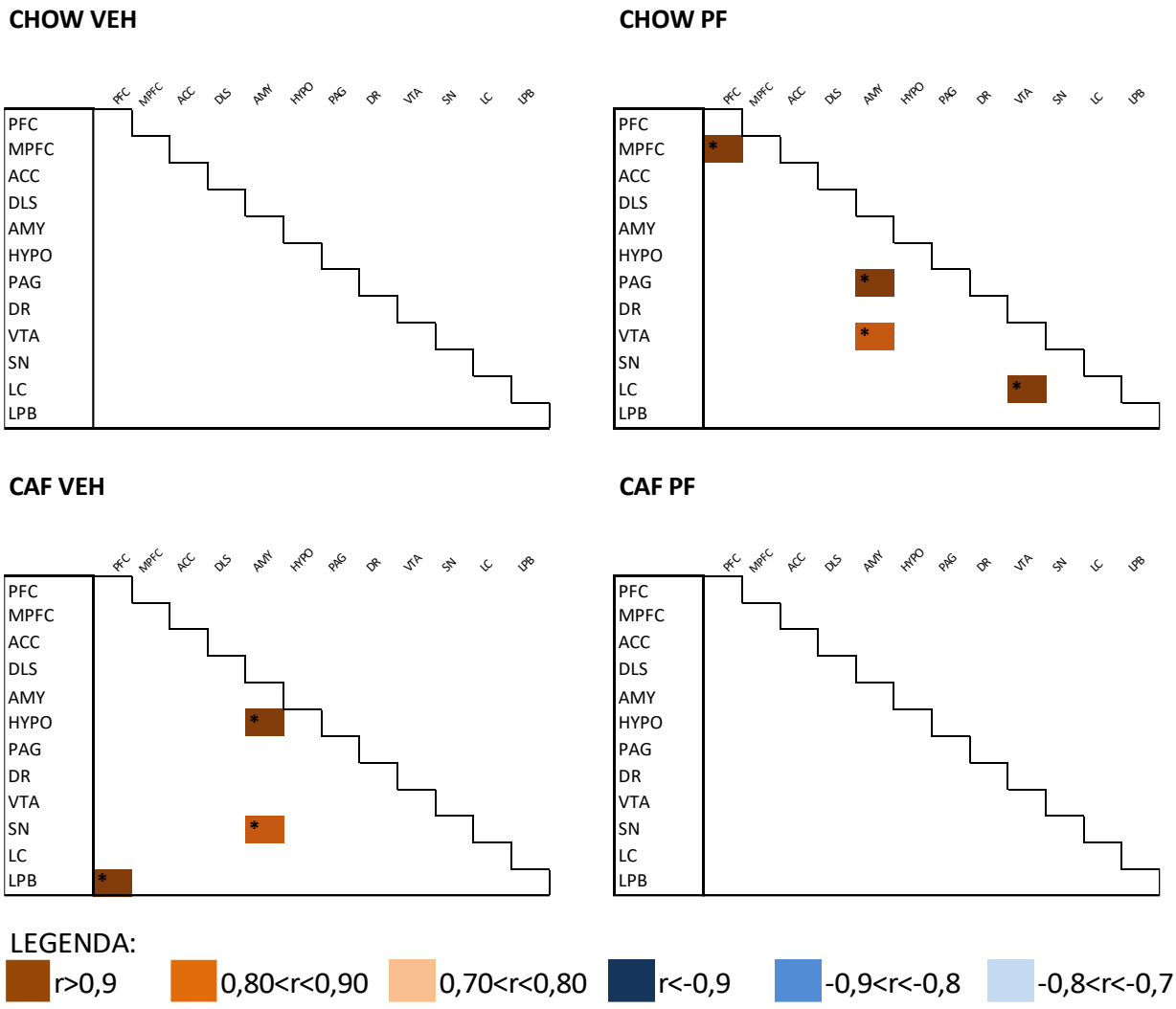

Fig.67: Pearson's correlation analysis results for NAPE-PLD. Only significant correlations are displayed (*p<0,05; **p<0,01).

DAGL alpha:

Differently from previous results, DAGL alpha correlation analysis revealed a reduction of the number of correlations in CAF VEH animals with respect to CHOW VEH ones. In addition, there was a variation of the areas which correlated to each other; at the same time while in CAF VEH animals the majority of correlations were negative, CHOW VEH animals displayed a majority of positive correlations. Once more, diet abstinence was capable of varying the functional interconnection between the different areas. On the other hand, PF-3845 treatment in rats experiencing diet withdrawal was not able to increment the number of correlations but re-established positive correlations throughout areas and results in a pattern of correlation more superimposable to the "normal" condition. Figure 68 shows the pattern of correlation for DAGL alpha the different brain areas analysed.

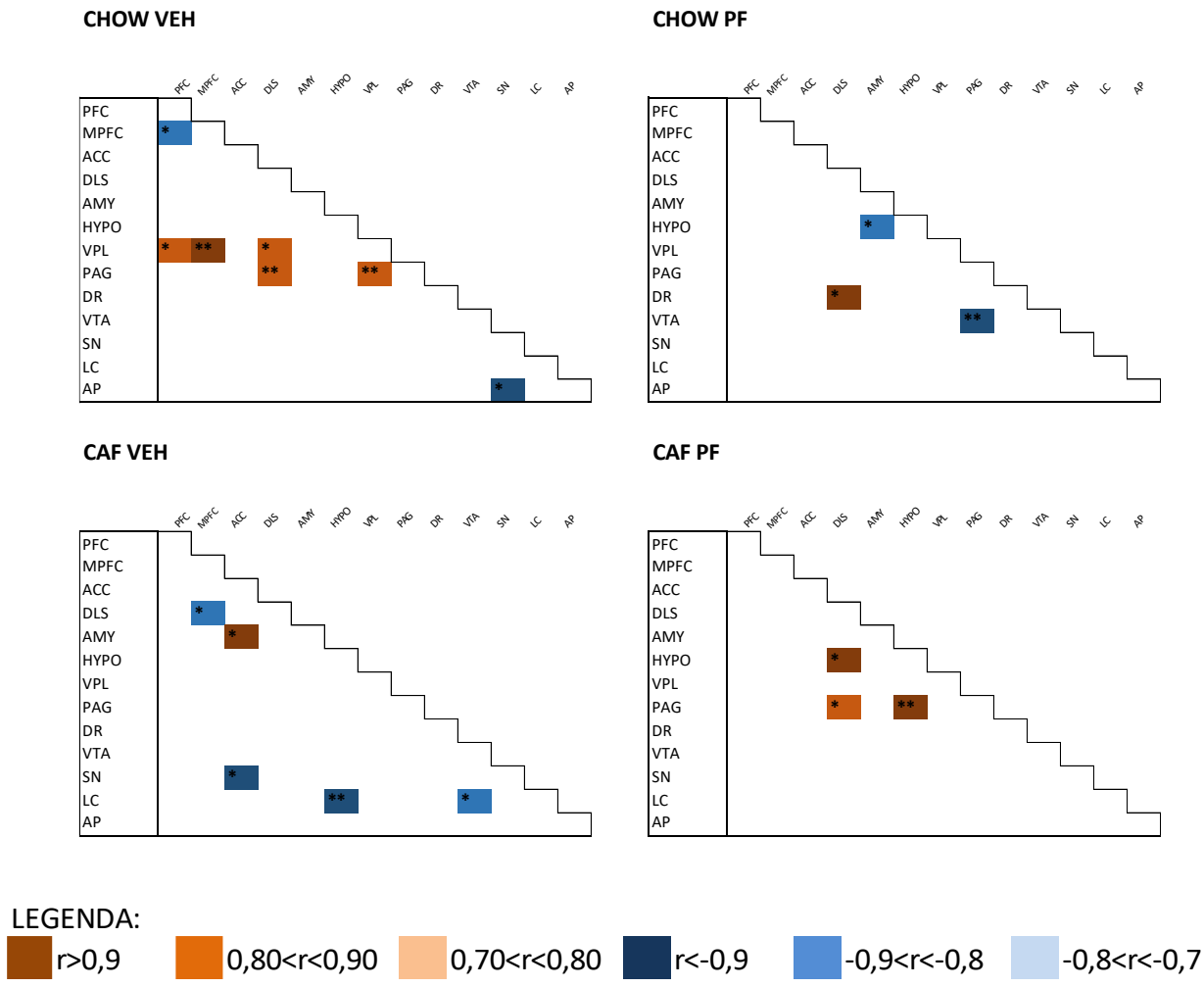

Fig.68: Pearson's correlation analysis results for DAGL alpha. Only significant correlations are displayed (*p<0,05; **p<0,01).

DAGL beta:

DAGL beta correlation analysis was affected by diet withdrawal: although the number of correlations was the same in CAF VEH animals and in CHOW VEH ones, the type of correlations was completely different and the correlation pattern isn't superimposable. Also in this case, PF-3845 administration wasn't able to normalize the pattern in CAF PF animals.

Figure 69 shows the pattern of correlation for DAGL beta in the different brain areas analysed.

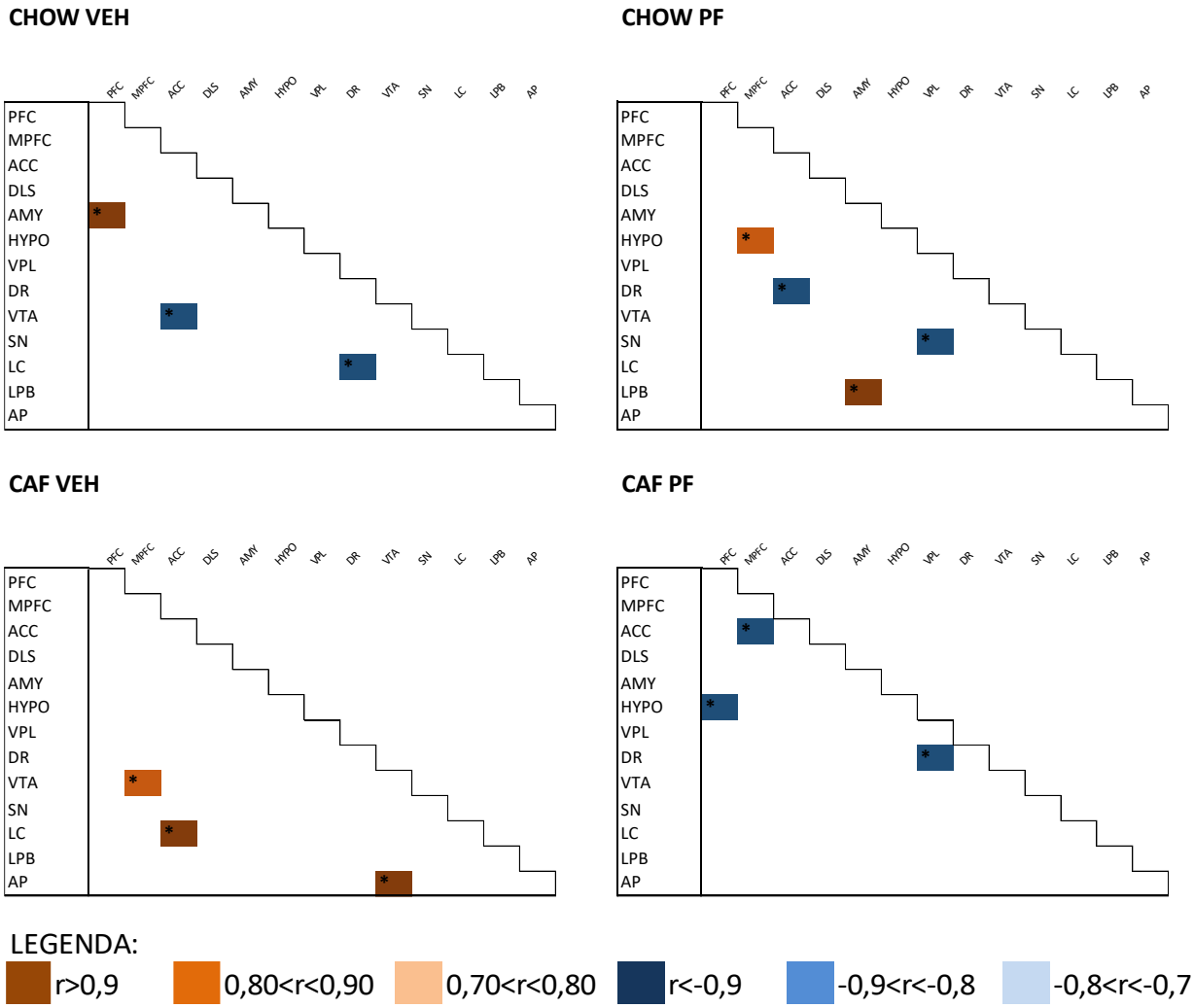

Fig.69: Pearson's correlation analysis results for DAGL beta. Only significant correlations are displayed (*p<0,05; **p<0,01).

GFAP:

Diet withdrawal was able to increase the number of in-between area correlations in CAF VEH animals. Pharmacological treatment with PF-3845 in CAF PF animals was not able to reduce the number of correlations, but at the same time, it varied the functional interconnections between areas, since it caused the development of a different pattern. It's important to notice how PF-3845 administration per se, in animals not exposed to the diet, was able to increase significantly the number of correlations, suggesting that pharmacological treatment can impact on the expression of this protein of inflammation. Figure 70 shows the pattern of correlation for GFAP in the different brain areas analysed.

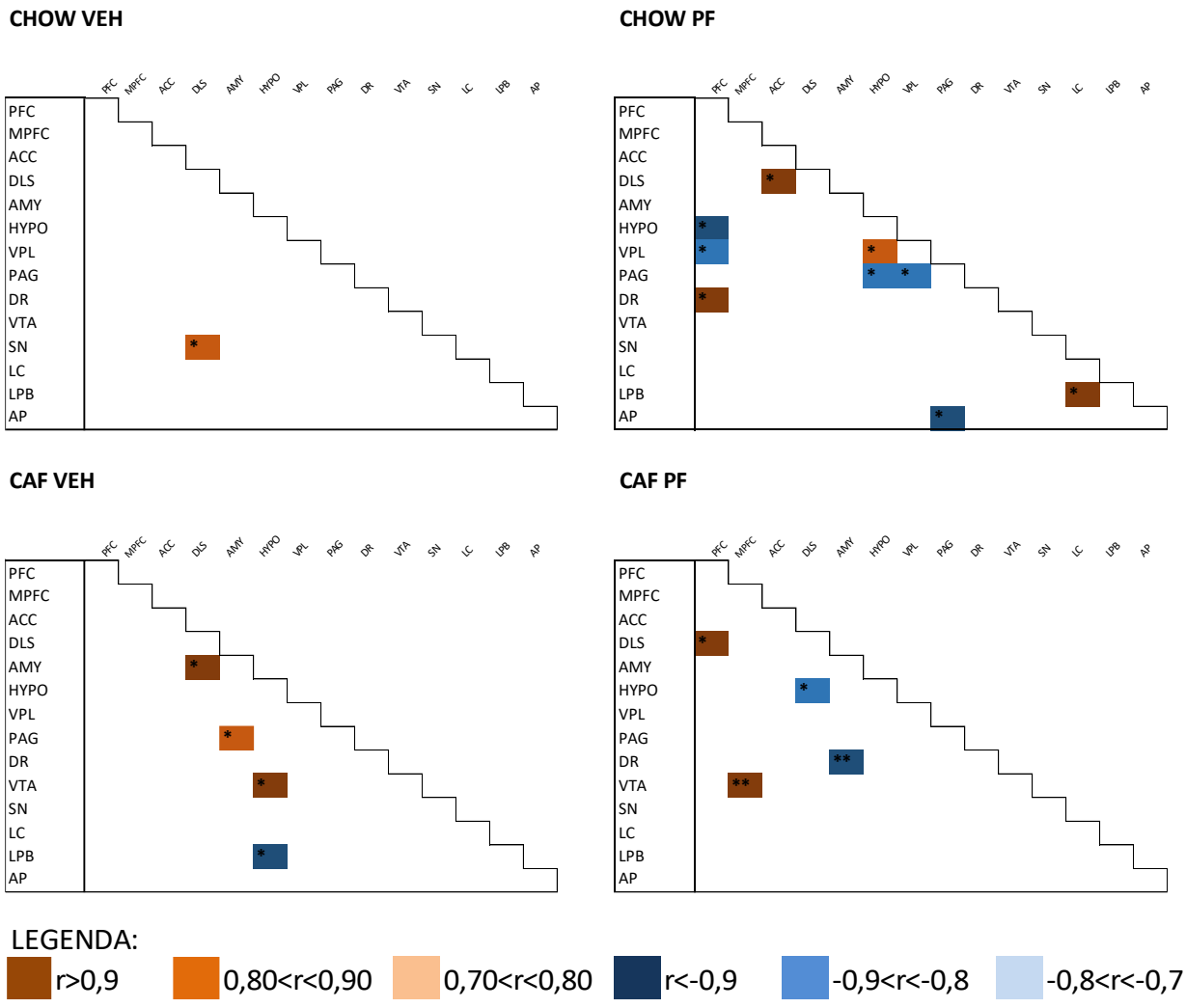

Fig.70: Pearson's correlation analysis results for GFAP. Only significant correlations are displayed (*p<0,05; **p<0,01).

IBA-1:

Also in this case it was possible to observe variations in the correlation pattern due to diet abstinence: CAF VEH animals showed a different pattern compared to CHOW VEH ones; once more PF-3845 treatment in CAF PF animals was not able to restore the pattern to a “normal” one.

Figure 71 shows the pattern of correlation for IBA-1 in the different brain areas analysed.

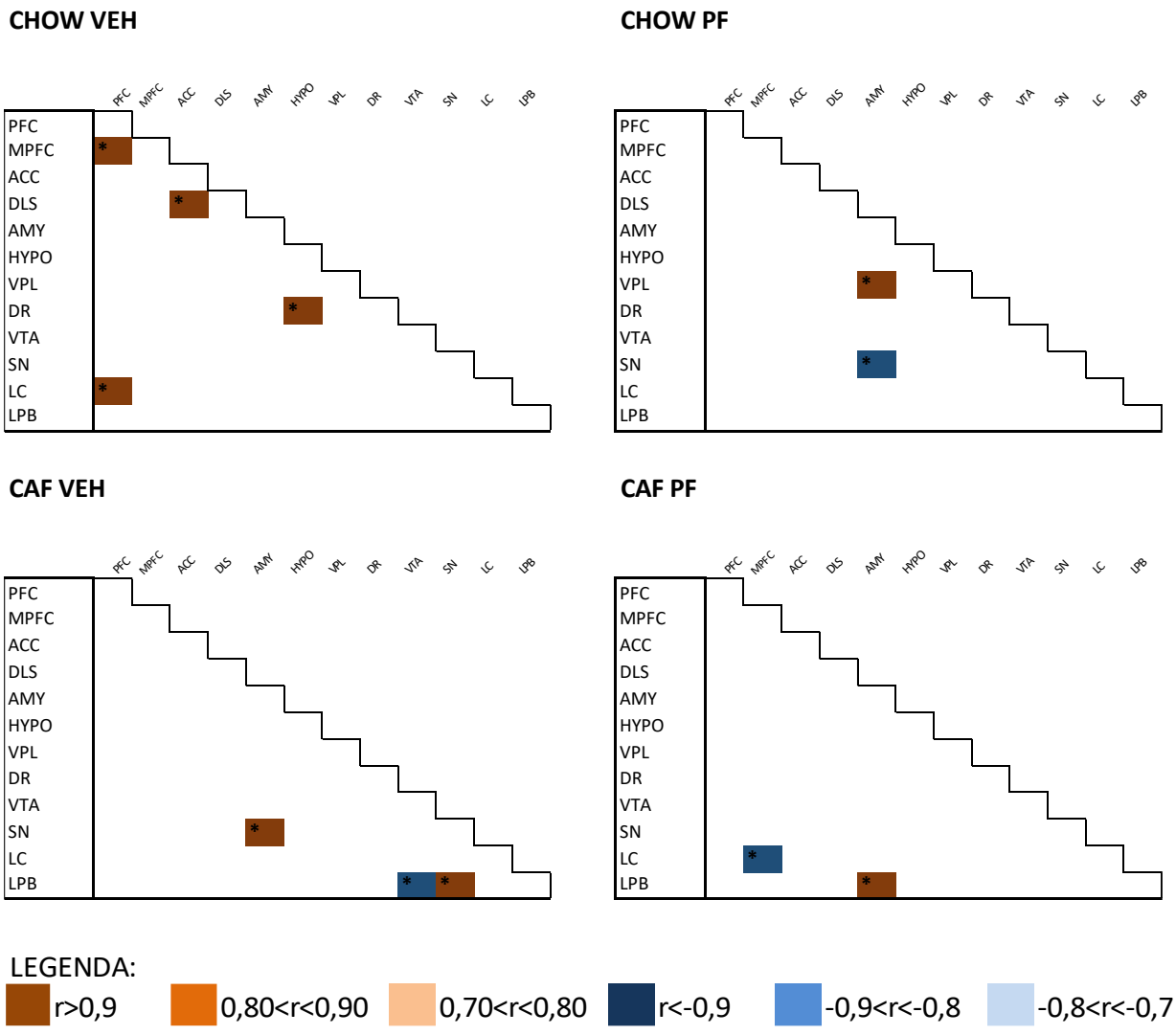

Fig.71: Pearson’s correlation analysis results for IBA-1. Only significant correlations are displayed (*p<0,05; **p<0,01).

6.4 CORRELATIVE ANALYSIS OF MONOAMINE AND CANNABINOID SYSTEM COMPONENTS IN THE SAME BRAIN REGION:

To test for the existence of a selective relationship between monoamine levels and endocannabinoid system proteins, we measured the coefficient of correlation between the monoamine content and protein expression of endocannabinoid system components in each brain area. Pearson correlation analysis was performed for each area separately, and the results for each experimental group were compared.

PREFRONTAL CORTEX:

In PFC, CAF VEH animals displayed a different pattern of correlation when compared to CHOW VEH group: we observed the loss of a correlation between NA and 5HT. At the same time, DA correlated with CB1 and CB2 and 5HT with CB1, thus suggesting that dopaminergic and serotonergic control of the endocannabinoid system receptors may vary due to diet abstinence. GFAP correlation with DAGL-beta varied from positive in CHOW VEH animals to negative in CAF VEH ones, thus suggesting that the endocannabinoid system could be involved in varying glial response in animals abstinent from the diet. Consistent variation of in-between correlation of enzymes of synthesis and degradation of the endocannabinoid system were observed between CAF VEH and CHOW VEH group. Administration of PF-3845 in CAF PF animals was partially able to restore a pattern similar to CHOW VEH animals, as we can observe a reduction of the number of the correlations and a variation of them. Figure 72 shows the different patterns of correlation developed in PFC for each experimental group.

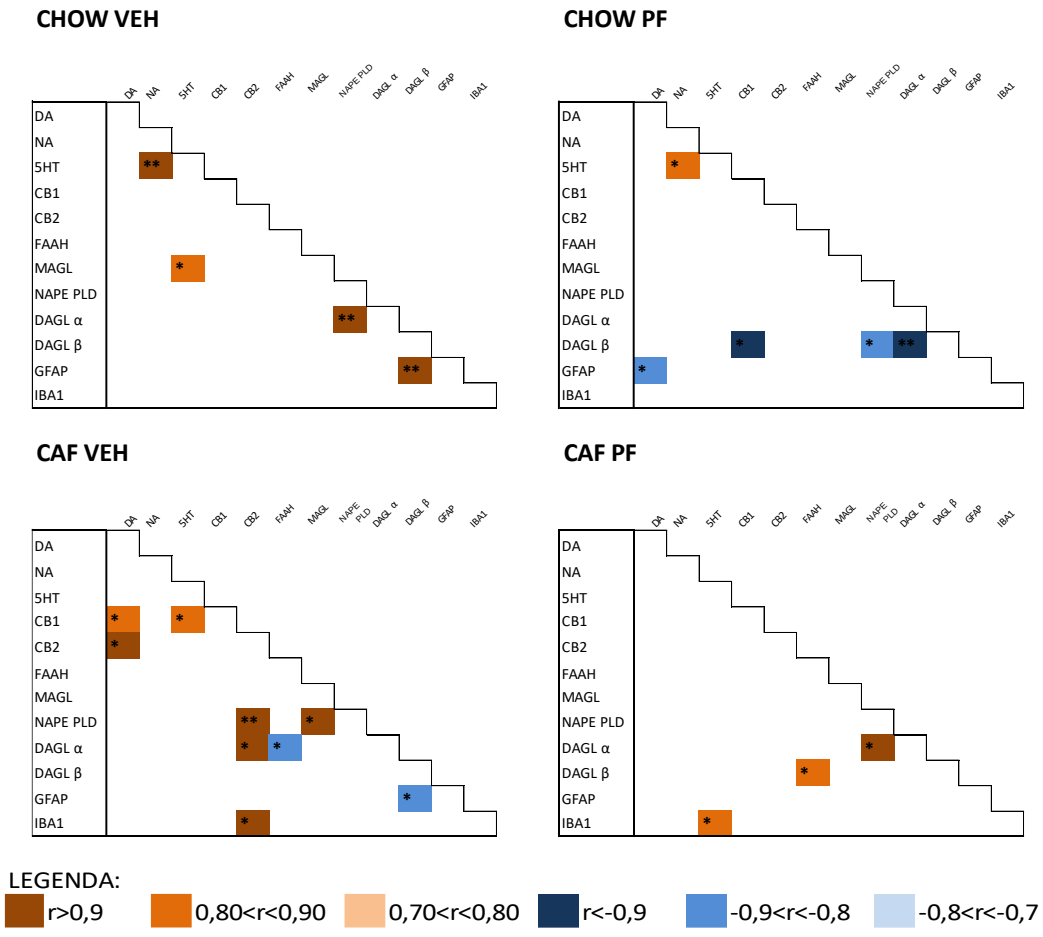

Fig.72: Pearson's correlation analysis results for PFC. Only significant correlations are displayed. *p<0,05; **p<0,01.

MEDIAL PREFRONTAL CORTEX:

Pearson correlation analysis in mPFC revealed that diet withdrawal might affect the interaction between monoamines (in particular, DA concentrations correlated with the ones of NA and 5HT in CAF VEH group; suggesting an interconnection between the systems absent in control animals) and at the same time also modified the interactions between the different components partaking to the endocannabinoid system. Moreover, in the same group, we were able to identify a negative correlation between GFAP expression and DA, thus suggesting that the monoamine could be involved in varying glial activation in this region in animals abstinent from the diet. Pharmacological treatment with PF-3845 was able to affect the aberrant pattern developed in CAF VEH animals in this region, but at the same time, this pattern appeared different from that observed in control group. Figure 73 shows the different patterns of correlation developed in mPFC for each experimental group.

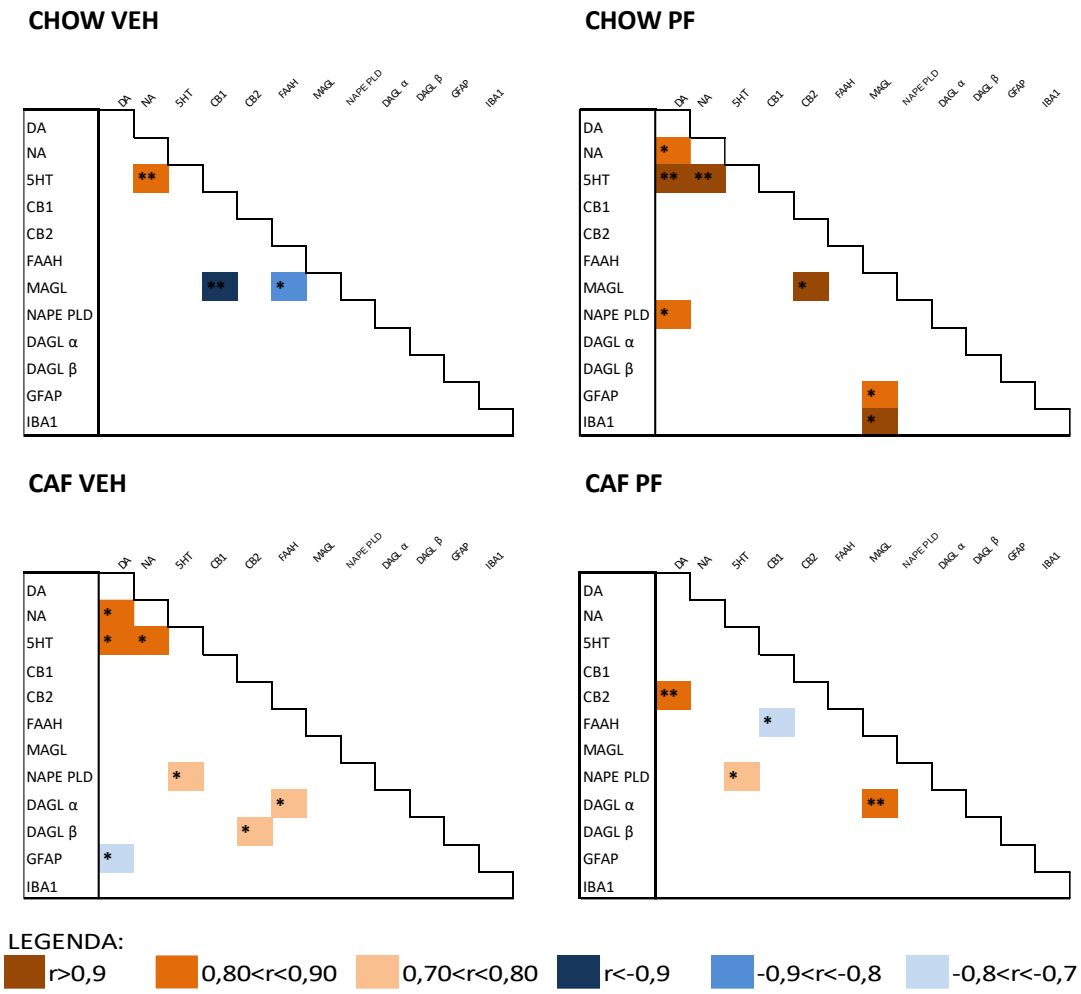

Fig.73: Pearson's correlation analysis results for mPFC. Only significant correlations are displayed. *p<0,05; **p<0,01.

NUCLEUS ACCUMBENS:

Similarly to what happened in mPFC, also in ACC diet withdrawal caused significant variations of the patterns of correlation. In fact, in CAF VEH animals, compared to CHOW VEH ones, there was the loss of the correlation between DA and 5HT in favour of a correlation between NA and 5HT. There was also a variation in the interconnection between the endocannabinoid system and monoamines since DA correlated with MAGL and NAPE-PLD in CAF VEH; differently from the CHOW VEH group. At the same time in CAF VEH animals, also the parameters which correlate with neuroinflammatory proteins (IBA-1 and GFAP) varied if compared with CHOW VEH group. PF-3845 treatment, in CAF PF group, could partially recover some of these variations, in particular the ones affecting GFAP and IBA-1 to a pattern similar to CHOW VEH group. Figure 74 shows the different patterns of correlation developed in ACC for each experimental group.

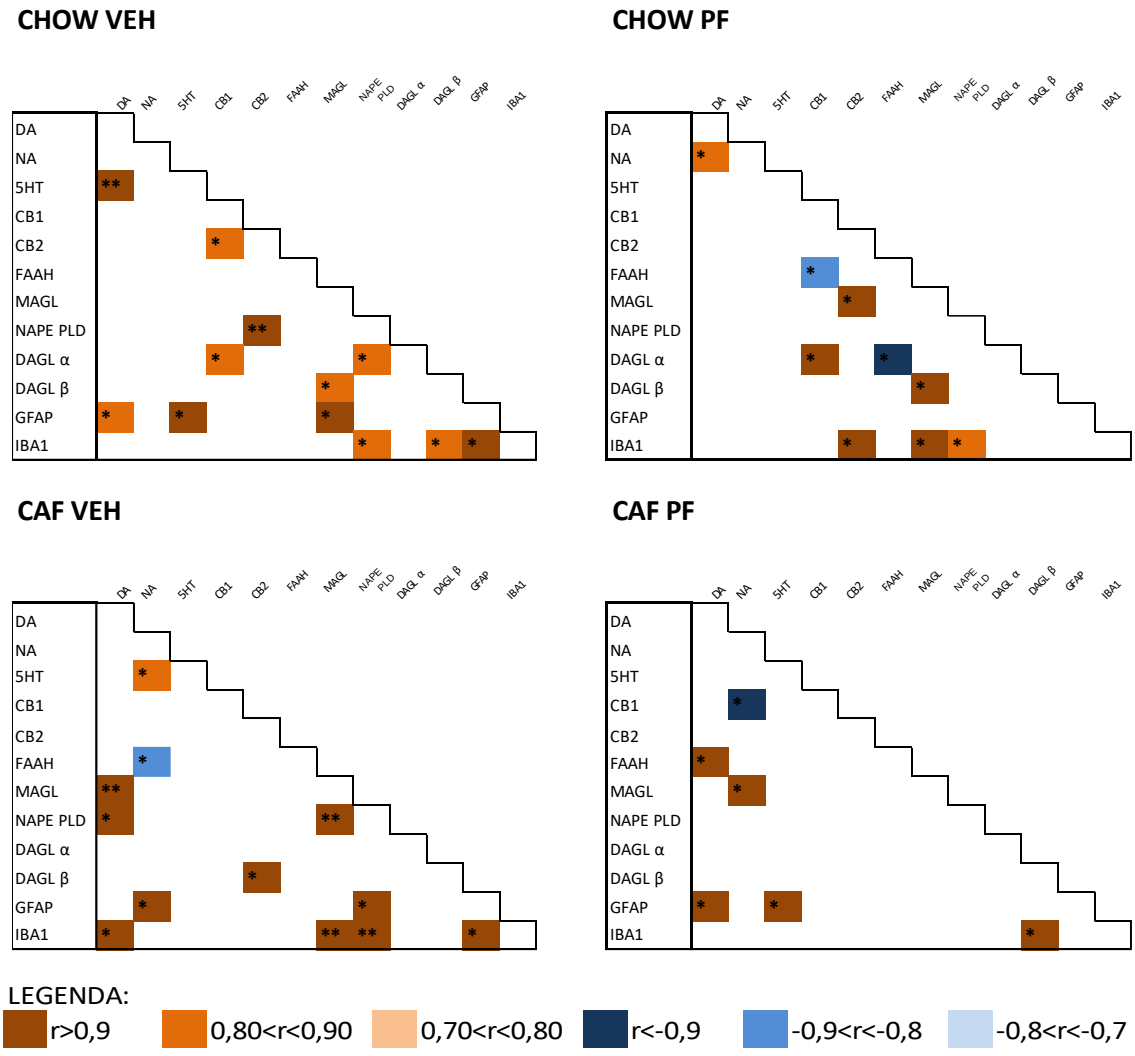

Fig.74: Pearson's correlation analysis results for ACC. Only significant correlations are displayed. *p<0,05; **p<0,01; ***p<0,001.

DORSOLATERAL STRIATUM:

Differently from what happened in the other areas, in DLS we observed a reduction of the number of correlations in CAF VEH animals, when compared to CHOW VEH ones. In particular, the correlation between CB2, MAGL and FAAH were loss in CAF VEH animals. Concerning the monoaminergic systems, a new correlation between NA and 5HT was observed in CAF VEH animals and not in CHOW VEH ones. PF-3845 administration in CAF PF group was able to restore negative correlation between NAPE PLD and IBA-1 in animals abstinent from the diet; and caused the presence of correlations between CB1, MAGL and FAAH; differently from CHOW VEH group. Figure 75 shows the different patterns of correlation developed in DLS for each experimental group.

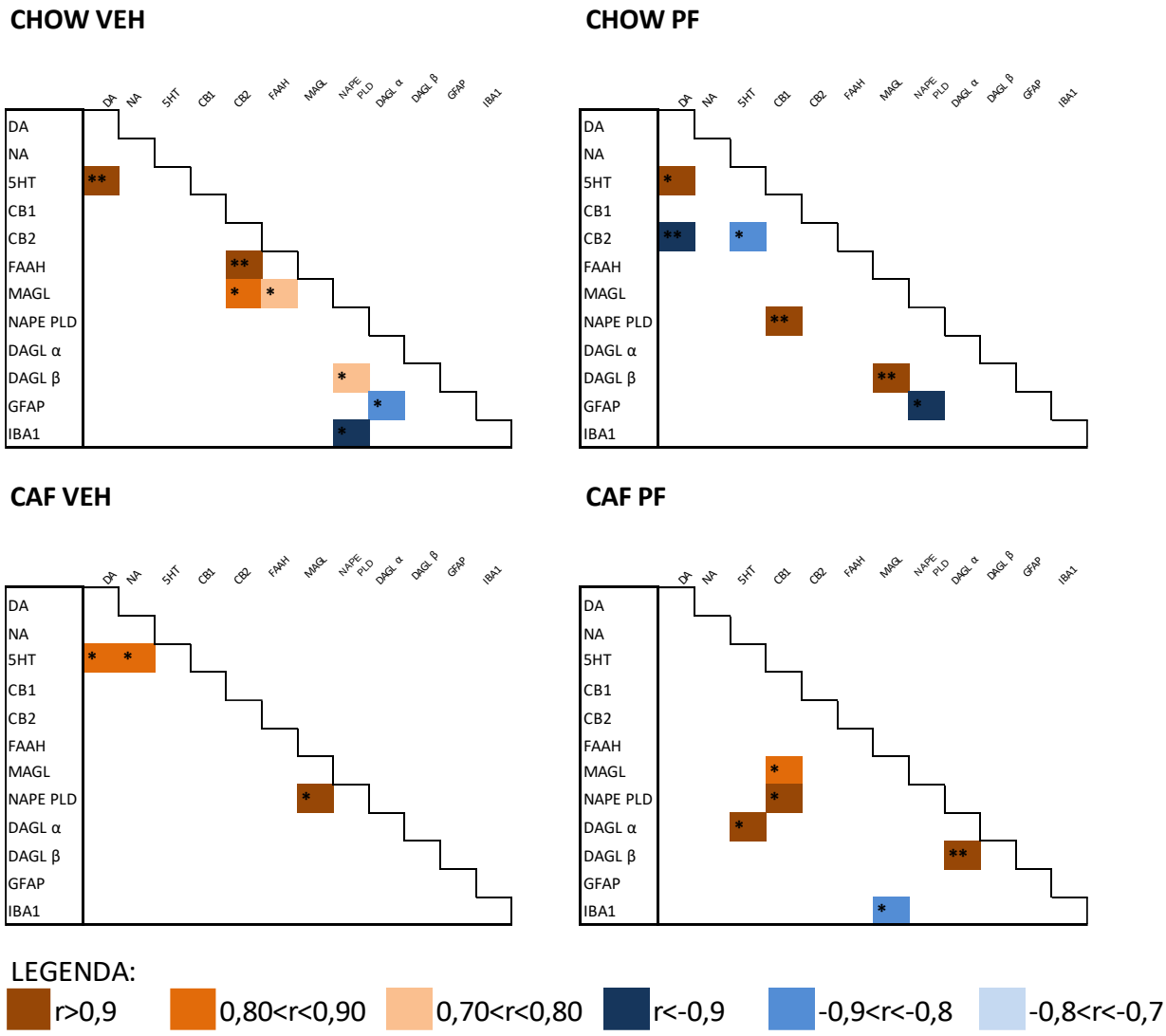

Fig.75: Pearson's correlation analysis results for DLS. Only significant correlations are displayed. *p<0,05; **p<0,01.

AMYGDALA:

Pearson's correlation analysis in AMY displayed significant variations of the correlations due to exposure and consequent abstinence from CAFETERIA diet. Likewise to what we observed in the other areas analysed, diet withdrawal affected the interactions between the monoaminergic systems. Pharmacological treatment with PF-3845 in CAF PF rats was able to restore the pattern of monoaminergic transmission to one similar to the control group and increase the number of correlations.

Figure 76 shows the different patterns of correlation developed in AMY for each experimental group.

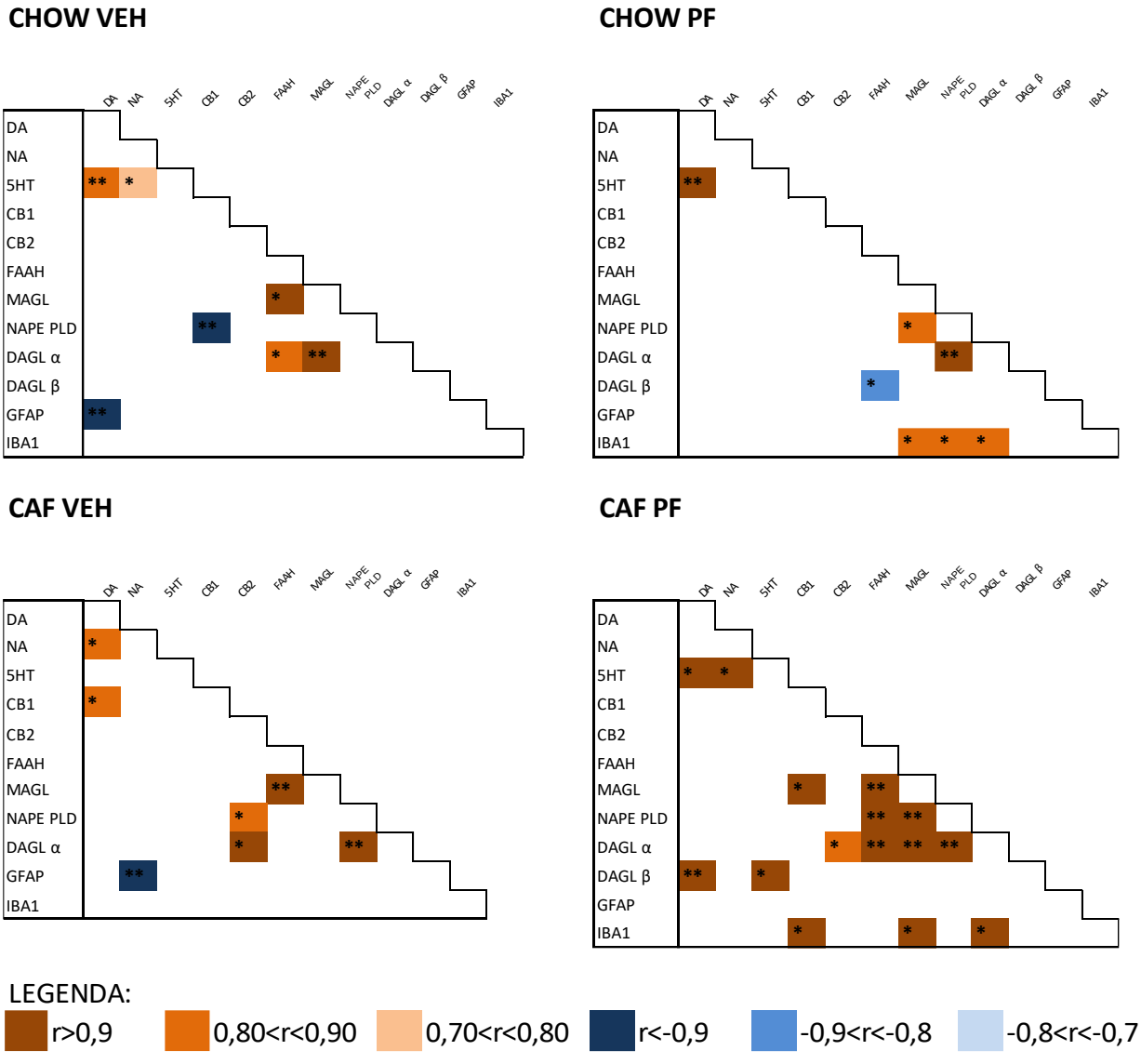

Fig.76: Pearson's correlation analysis results for AMY. Only significant correlations are displayed. *p<0,05; **p<0,01.

HYPOTHALAMUS:

The analysis of correlations in HYPO showed that diet abstinence could lead to a decrease in the number of total correlations; especially the ones that involve the endocannabinoid system. Once more, diet withdrawal caused differences in the pattern of correlation of monoamines, in particular animals abstinent from the diet showed a correlation between NA and 5HT in this area, which is absent in the control group. Pharmacological treatment with PF-3845, in this case, was not effective in restoring a pattern similar to the control group; even if it was effective in increasing the number of correlations of proteins partaking to the endocannabinoid system. Figure 77 shows the different patterns of correlation developed in HYPO for each experimental group.

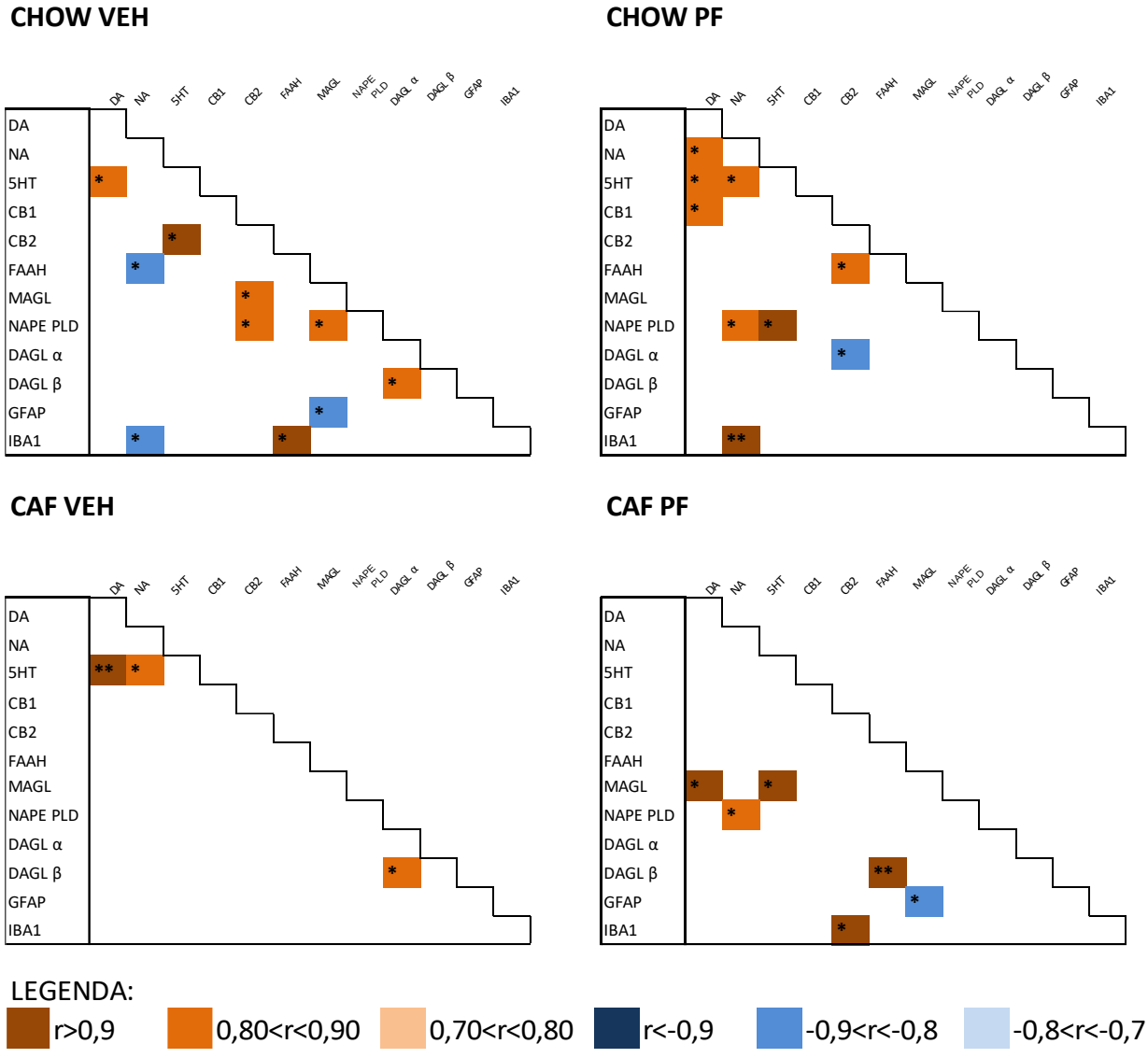

Fig.77: Pearson's correlation analysis results for HYPO. Only significant correlations are displayed. *p<0,05; **p<0,01.

VENTRAL PALLIDUM:

CAF VEH animals once more show a different pattern from CHOW VEH, thus suggesting that diet abstinence was able to affect the patterns of correlation between proteins partaking to the endocannabinoid system and monoamines. In particular, in VPL CAF VEH animals lost a correlation between 5HT and DA. It is important to notice how diet abstinence influenced the pattern of correlation of IBA-1: in CAF VEH animals IBA-1 correlated positively with DA and negatively with FAAH; whereas in CHOW VEH animals it's shown a positive correlation between IBA-1 and CB2, FAAH and GFAP. Pharmacological treatment with PF-3845 in CAF PF animals was not able to create a pattern superimposable to a "normal" condition; but influenced the correlations of GFAP. Figure 78 shows the different patterns of correlation developed in VPL for each experimental group.

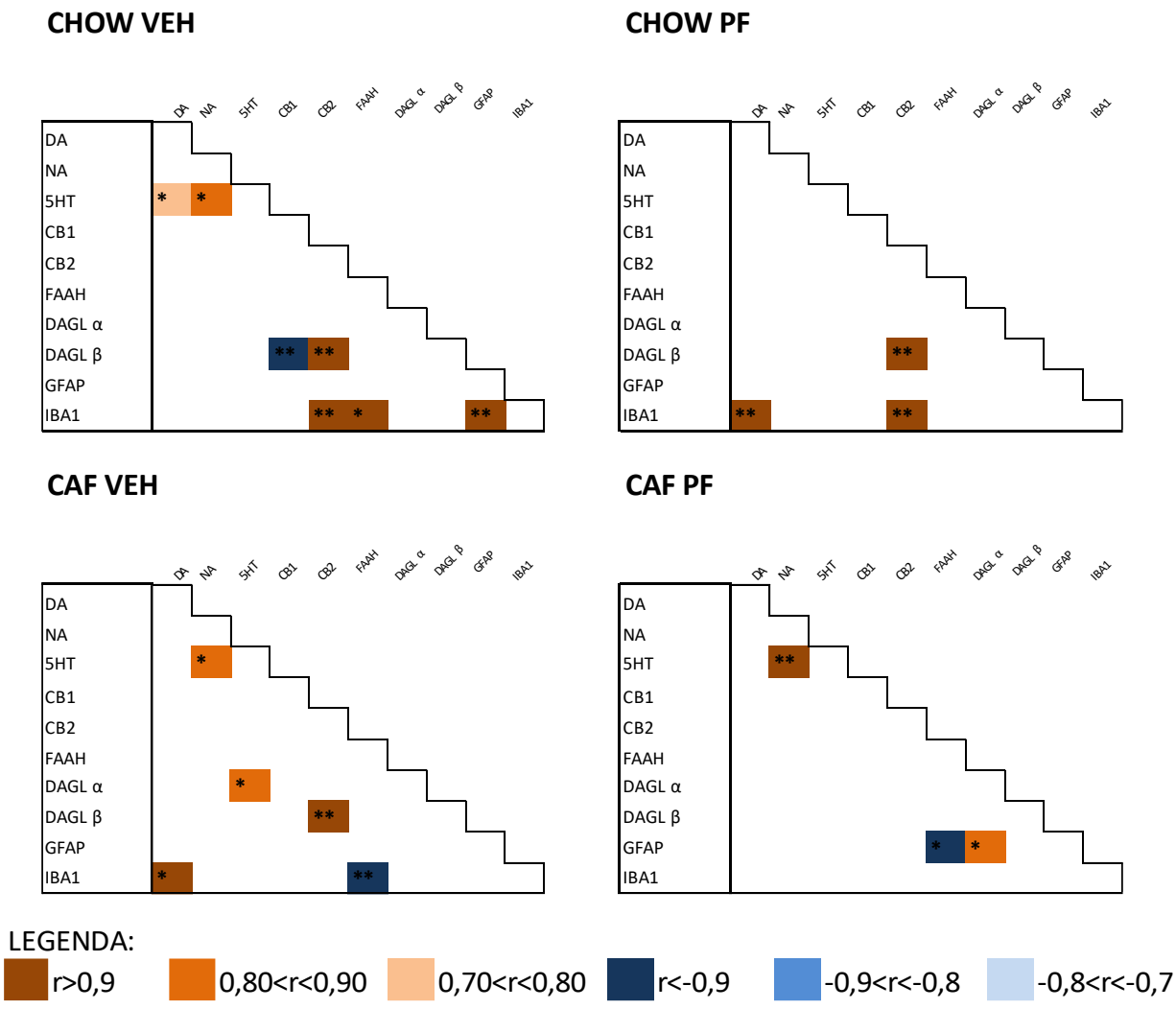

Fig.78: Pearson's correlation analysis results for VPL. Only significant correlations are displayed. *p<0,05; **p<0,01.

PERIACQUEDUCTAL GREY:

CHOW VEH animals showed a negative correlation between NAPE PLD and DAGL alpha. CHOW PF animals, differently from vehicle treated ones, showed a positive correlation between 5HT and NA and a negative correlation between CB1 and NAPE PLD. CAF VEH animals, after experiencing abstinence from the diet, displayed a negative correlation between 5HT and NA. CAF PF animals, differently from the vehicle treated ones, displayed a positive correlation between DA and 5HT. Figure 79 shows the different patterns of correlation developed in HYPO for each experimental group.

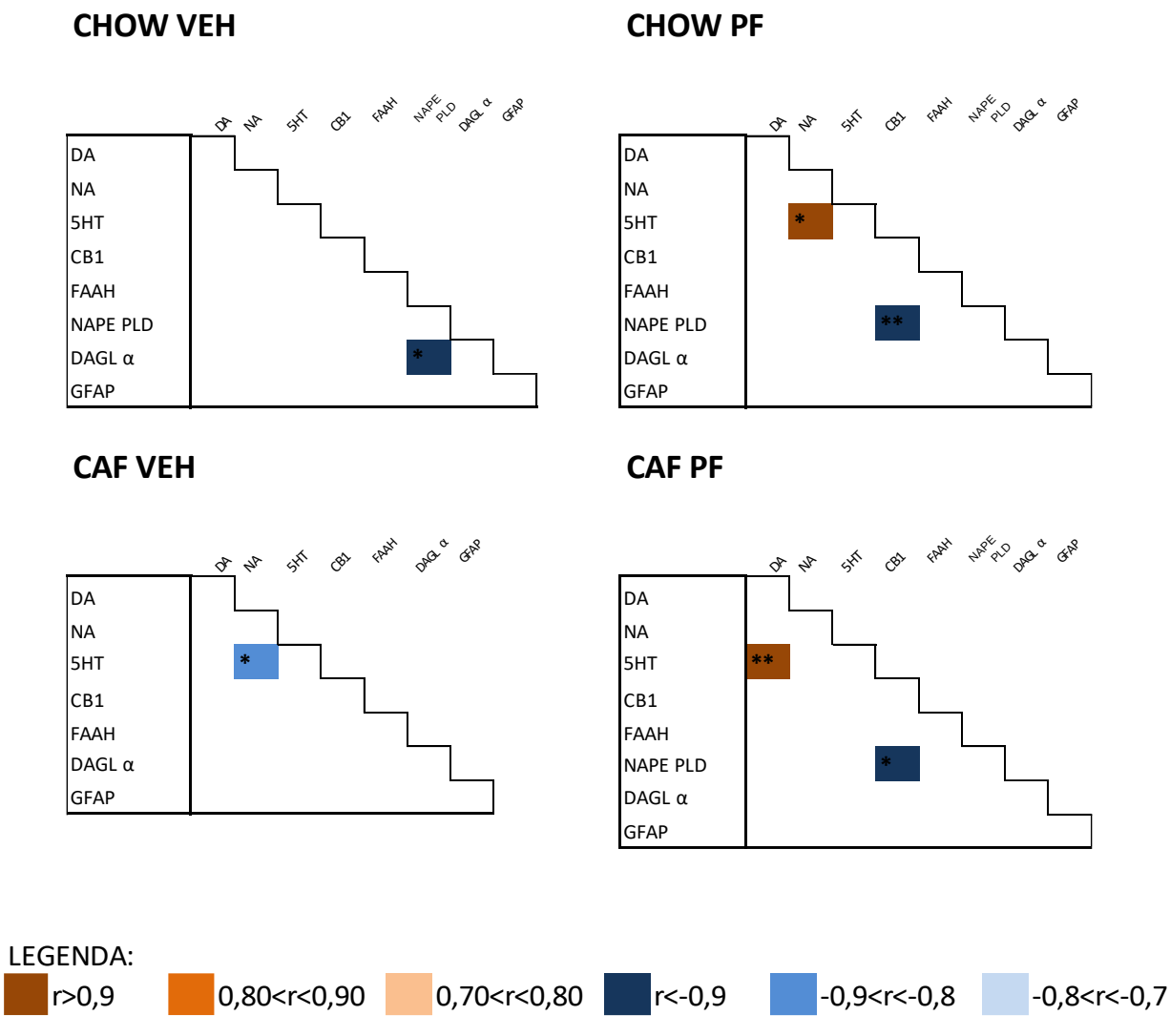

Fig.79: Pearson's correlation analysis results for PAG. Only significant correlations are displayed. *p<0,05; **p<0,01.

DORSAL RAPHE:

Regarding the correlations occurring in DR, we can notice how diet abstinence in CAF VEH group was not able to vary the correlations occurring between the different monoamines; although it affected a lot the correlations between the proteins of the endocannabinoid system, with the loss of some correlations between CB1, CB2 and FAAH. Most importantly we noticed in CAF VEH animals a negative correlation between NAPE and DAGL alpha; differently from CHOW VEH animals; where the same correlation was positive. Same variations occurred in the correlations between DAGL alpha and DAGL beta and DAGL alpha and IBA-1. Pharmacological treatment, in CAF PF animals; was partially able to restore the correlations to a pattern similar to CHOW VEH; referring to a correlation between CB1 and CB2 and the one between NAPE and DAGL alpha. Figure 80 shows the different patterns of correlation developed in DR for each experimental group.

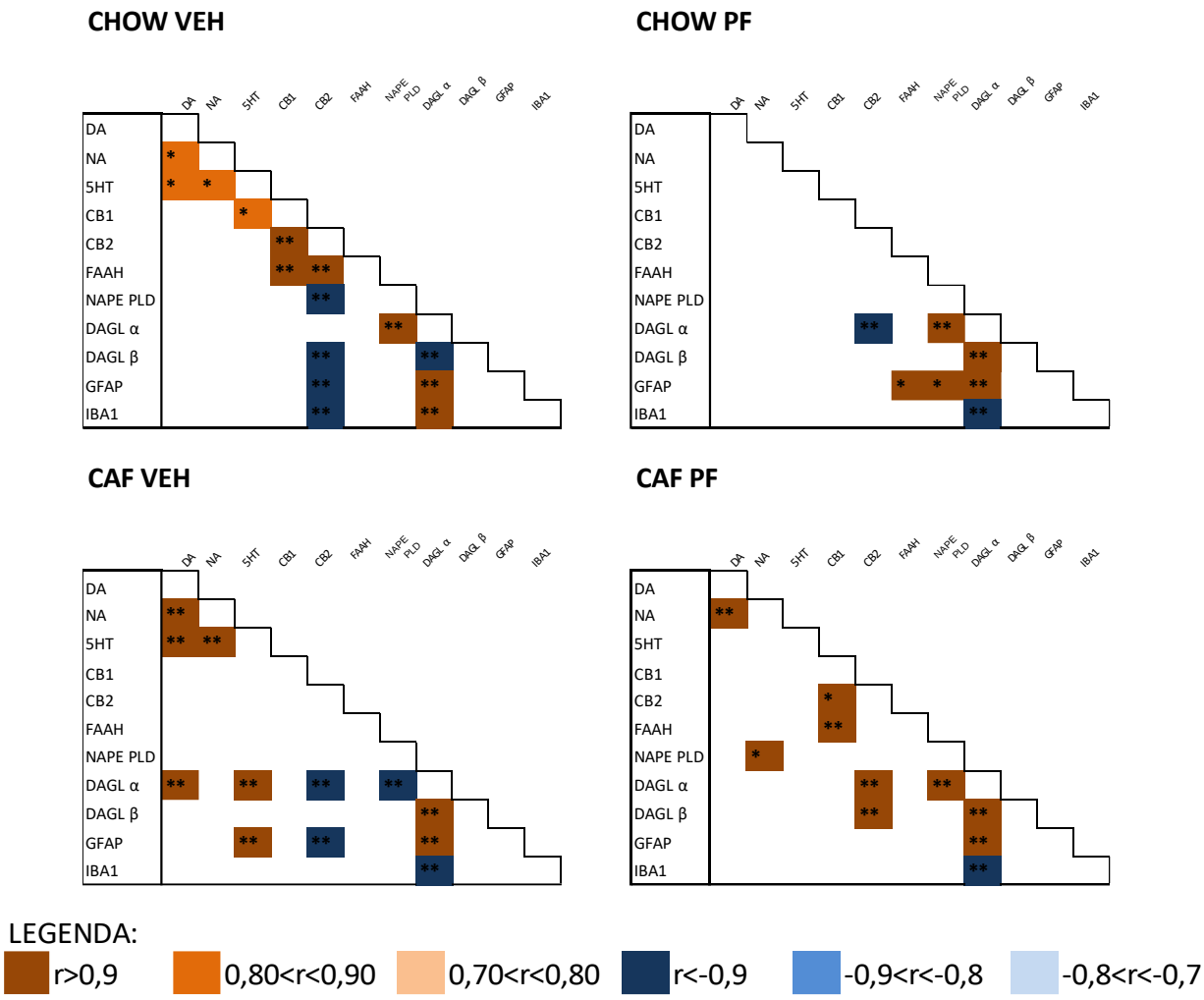

Fig.80: Pearson's correlation analysis results for DR. Only significant correlations are displayed. *p<0,05; **p<0,01; ***p<0,001.

VENTRAL TEGMENTAL AREA:

In VTA, similarly to what happened in the other areas, we observed that diet withdrawal was able to vary the correlations between monoamines. In particular, CAF VEH animals displayed a correlation between 5HT and DA; differently from CHOW VEH animals which displayed a correlation between 5HT and NA. Moreover, in CAF VEH animals DAGL-alpha was related to DA and 5HT concentrations; differently from the control group. Additionally, also the correlations regarding the microglial marker IBA-1 varied from the control group. In this area, treatment with PF-3845 acted on varying the correlation patterns of the endocannabinoid system, but the pattern developed still was completely different from the control group. Figure 81 shows the different patterns of correlation developed in VTA for each experimental group.

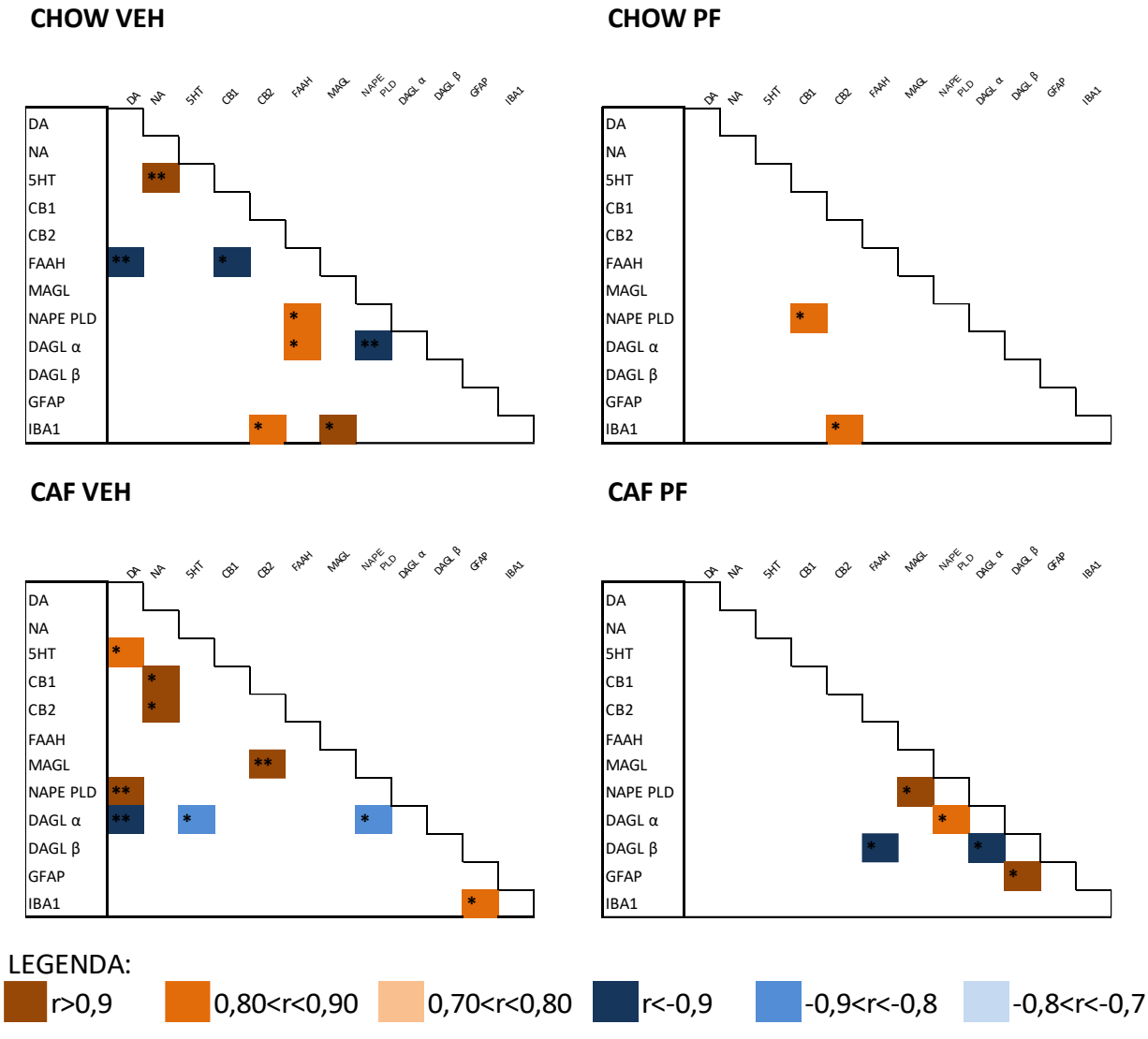

Fig.81: Pearson's correlation analysis results for VTA. Only significant correlations are displayed. *p<0,05; **p<0,01; ***p<0,001.

SUBSTANTIA NIGRA:

In SN, CAF VEH animals displayed a correlation between DA and NA that is absent in the control group. However, the most interesting data comes from correlations in CAF PF group: PF-3845 administration could restore the correlations between monoamines to the ones of the control group. At the same time pharmacological treatment was effective in creating a negative correlation between DA, NA, 5HT and CB2 and FAAH, thus suggesting that the monoaminergic control of the endocannabinoid system in this area could be involved in its mechanism of action and its effectiveness in reducing the response to abstinence. Figure 82 shows the different patterns of correlation developed in SN for each experimental group.

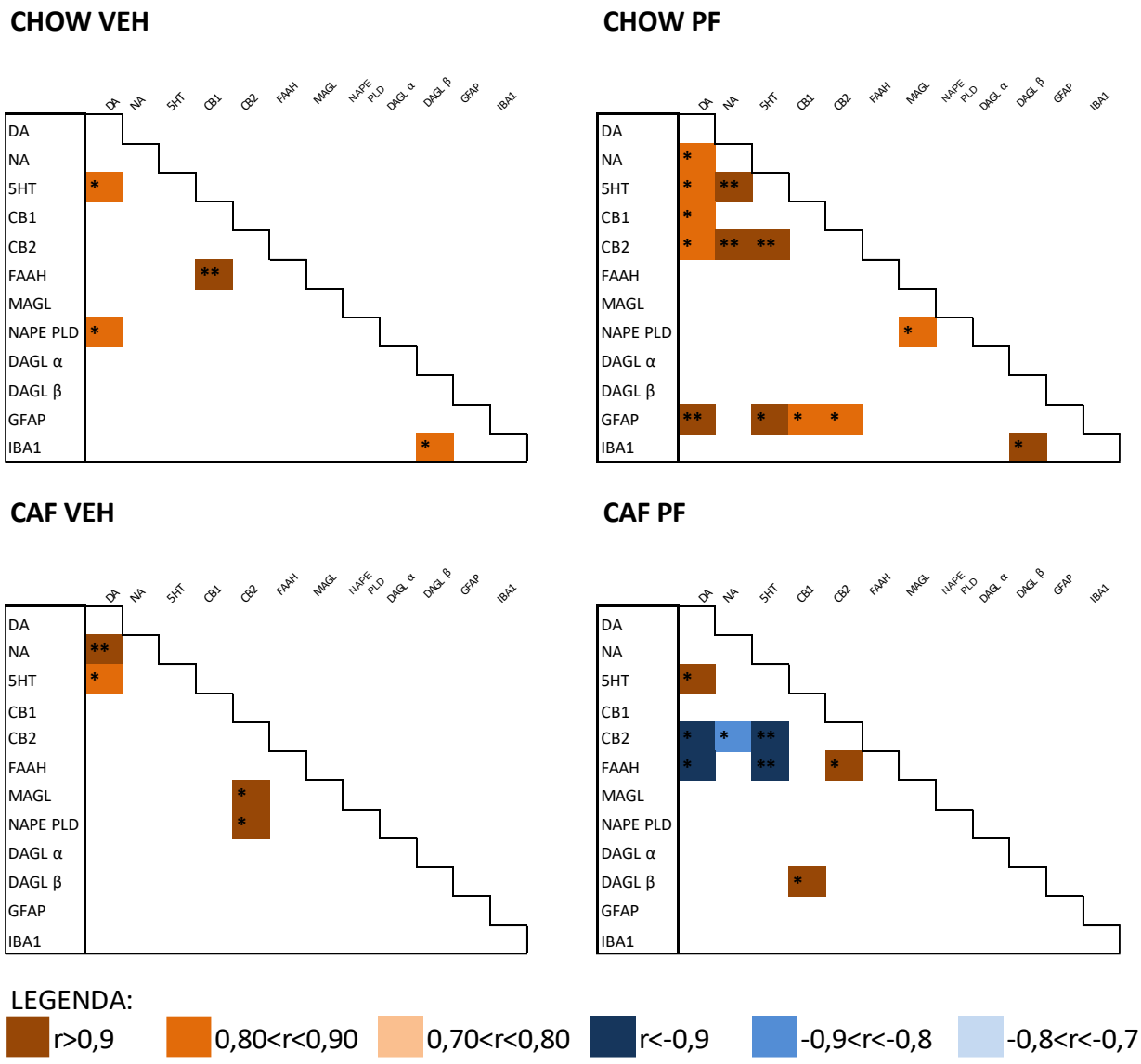

Fig.82: Pearson's correlation analysis results for SN. Only significant correlations are displayed. *p<0,05; **p<0,01.

LOCUS COERULEUS:

In LC, abstinence from CAFETERIA diet caused the loss of correlation between DA, NA and 5HT in CAF VEH rats when compared to CHOW VEH ones. Moreover, between the same groups, we could observe a loss of the correlation between NAPE PLD and NA and the presence of a negative correlation between NAPE PLD and 5HT. Pharmacological treatment, in animals abstinent from the diet (CAF PF) was able to establish the same pattern of correlation of CHOW VEH animals concerning monoamines. Moreover, this group displayed a negative correlation between DA, NA and CB1. Figure 83 shows the different patterns of correlation developed in LC for each experimental group.

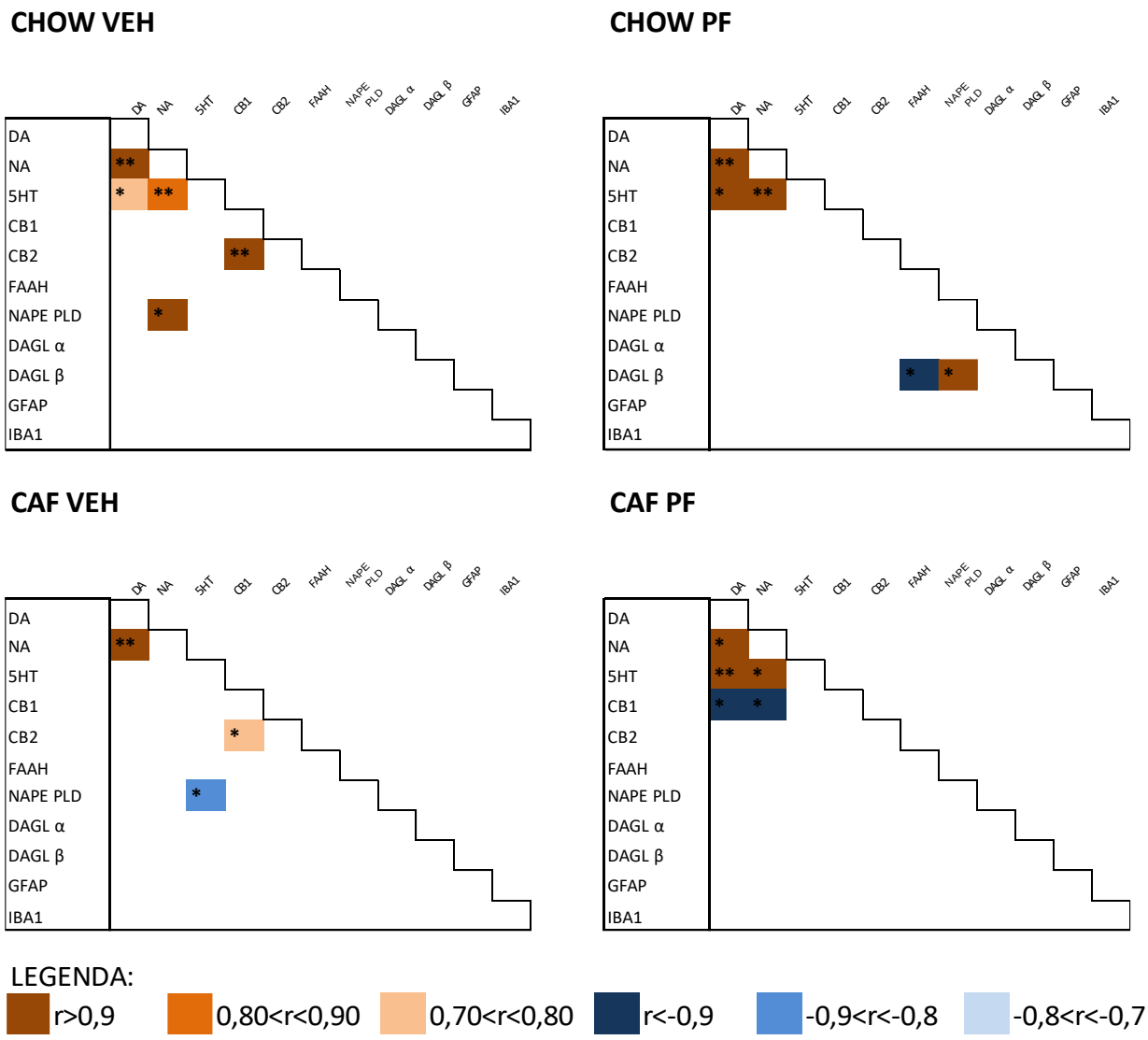

Fig.83: Pearson's correlation analysis results for LC. Only significant correlations are displayed. *p<0,05; **p<0,01.

LATERAL PARABRACHIAL:

Differently from the other areas, in LPB diet abstinence was not able to vary consistently the correlation pattern between monoamines and the endocannabinoid system, suggesting that this area was not involved in anxiety-like and depressive-like behaviours associated with diet abstinence. The only difference between CAF VEH animals and CHOW VEH ones was a negative correlation between DAGL beta and CB1 which is loss in CAF VEH group. On the other hand, pharmacological treatment with PF-3845 was able to increase the number of in-between correlations, both in animals experiencing diet withdrawal and naïve ones.

Figure 84 shows the different patterns of correlation developed in LPB for each experimental group.

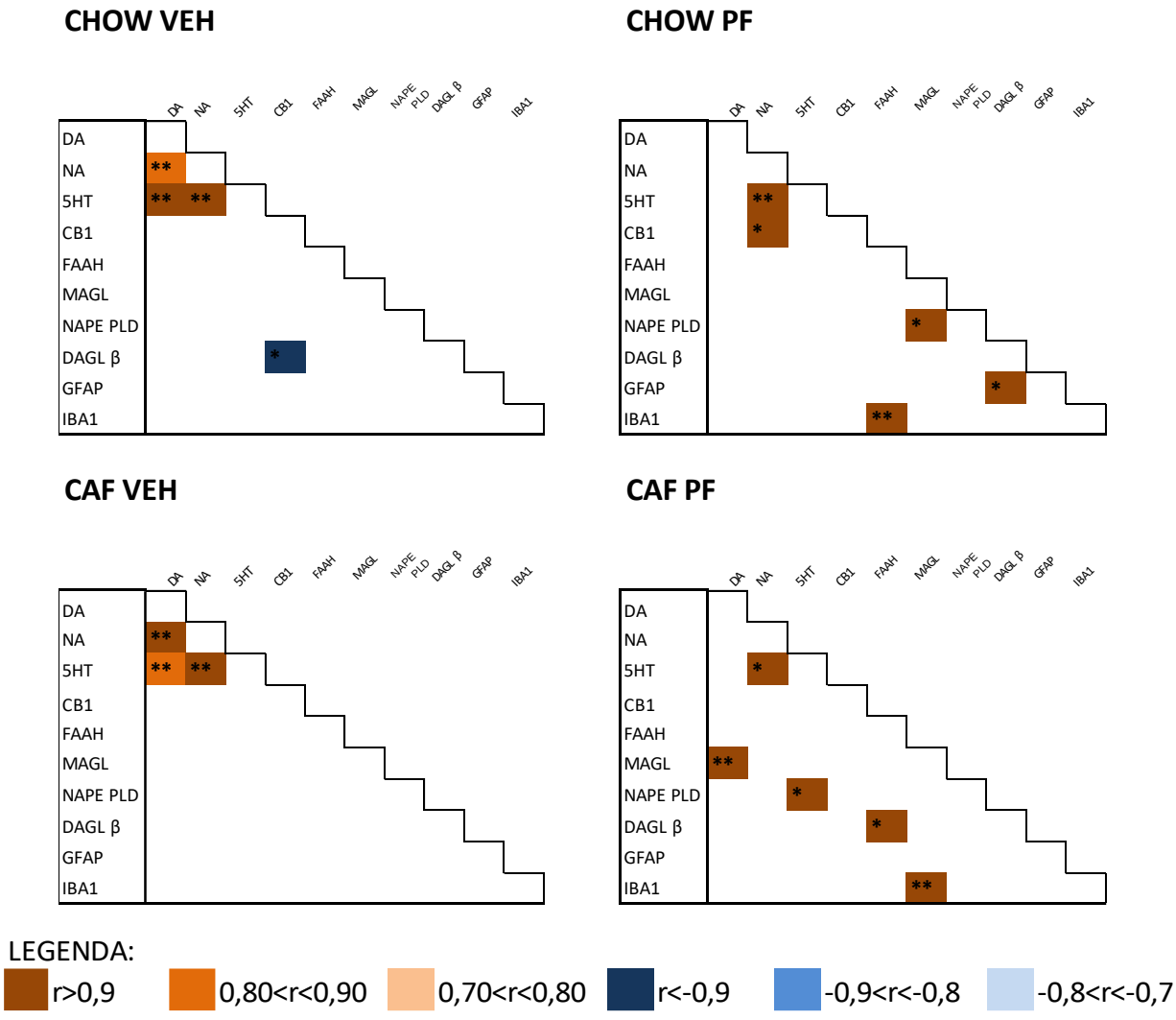

Fig.84: Pearson's correlation analysis results for LPB. Only significant correlations are displayed. *p<0,05; **p<0,01.

CHAPTER 4 – DISCUSSION

Our study demonstrates that 28-days abstinence from a palatable diet is able to affect mood and behaviour of rats and cause variations of the monoaminergic and endocannabinoid systems in various areas of the brain, thus suggesting that high-palatable food exposure for long periods of time can induce long lasting alterations in the brain that are not restored by weight loss, or by diet abstinence. Conversely, the pharmacological treatment with PF-3845 during the abstinence period is able to restore many of these alterations, thus supporting the hypothesis of a protective role played by anandamide and other acylethanolamides, whose edogenous levels can be increased by FAAH inhibition.

A first aspect of our research has been focused in investigating the variations occurring to the reward system: as widely explained in the introduction, hedonic obesity is characterized by reward system impairments[16], which is strictly interconnected with DA neurotransmission[194]. Dopaminergic transmission overview, given by Pearson correlation analysis of DA concentration and turnover in the different areas, shows that dopaminergic system is impacted by diet exposure and abstinence, since abstinent animals present different patterns of correlation with respect to chow-fed animals. This thesis is supported by our results, that demonstrate that although exposure and subsequent abstinence from cafeteria diet has no impact on DA synthesis in VTA and SN, since no significant variation has been observed in TH expression, neither in DA, DOPAC and HVA concentration or turnover in the two areas; it can affect DA transmission in the projecting areas, such as ACC and DLS. A significant reduction of TH immunostaining has been detected in these areas in abstinent rats and means reduction of DA projections, which is compatible with several studies sustaining dopaminergic system hypoactivation in obese individuals[274], although these variations are not accompanied by significant variation of DA, DOPAC, HVA amounts or DA turnover.

Cafeteria diet abstinence impacts also the endocannabinoid system in these areas, inducing variations that are compatible with the previous data: in VTA we notice a decreased CB2 expression, which can be linked to reduced DA neuronal excitability[275] and consequently reduced reward response. In the same region, cafeteria-diet abstinent rats present an increased expression of FAAH, resulting in altered endocannabinoid concentration and

consequent variation of hedonic feeding behaviour, food preferences, motivation and reward associated with consuming foods[276]. On the other hand, NAPE-PLD decreased expression in SN can be responsible of reduced acylethanolamides production, decreased neuroprotective action and dampened survival of SN dopaminergic cells and projections[277], whereas increased CB1 expression in this area has been linked with addiction and withdrawal[278]. Other evidence of impaired reward activity and addiction comes from ACC: abstinence from the diet causes CB1 receptor decrement and dampens the rewarding effects of DA, since CB1 in this region activates mesolimbic DA reward circuitry[279,280]; whereas CB2 decreased activity[281,282] and DAGL alpha expression variations[283] can be a sign of addictive behaviour. Furthermore, correlative analysis between monoamines and endocannabinoid system in these dopaminergic areas evidence that diet abstinence is able to vary the patterns of correlations with respect to naïve animals, suggesting an impairment of the interconnection between monoamines and cannabinoids. In particular, the reduced number of correlations observed in DLS of abstinent rats (when compared to chow-fed ones) suggests an hypoactivation of this area, that has been evidenced also in other studies regarding obesity[36,117,204].

Even though pharmacological treatment with PF-3845 is not able to affect TH expression in VTA, SN, ACC and DLS, it successfully increases DA concentration in DLS, promoting dopaminergic transmission in this area, that is drastically impacted by obesity[36,117,204]. At the same time, drug administration is able to increase 5HT concentration in SN, and promote DA transmission (in fact, 5HT agonists administration in this region have been shown to increase DA dendritic efflux[284]). As a consequence, PF-3845 administration in animals abstinent from the diet is also able to increment the correlations number of DA and DA turnover among the different regions when compared to vehicle treated abstinent rats, sign of a stimulated DA transmission[285]. At the same time, we notice an increased number of correlations between monoamines and endocannabinoid system proteins in SN and DLS of abstinent treated animals when compared to vehicle-treated ones, suggesting increased nigrostriatal pathway activation. Moreover, PF-3845 administration reduces the activation of the mesolimbic pathway (as evidenced by the decreased number of correlations in VTA and ACC of abstinent treated animals when compared to vehicle-treated ones); thus suggesting that the pharmacological treatment can restore a normal striatal activity and reduce the mesolimbic hyperactivation in response to food cues[8]. At the same time, PF-3845 is able to partially

restore the alterations detected in the endocannabinoid system, in particular decreasing the expression of FAAH in VTA and of CB1 in SN. Moreover, PF-3845 administration in animal subjected to the withdrawal reduces CB1 expression and increases CB2 expression in PFC: both increased CB1 and decreased CB2 expression in this area have been linked with addiction[286], confirming the role of PF-3845 in re-establishing reward and addicted behaviours.

Besides VTA, SN, DLS and ACC, that are the most important areas involved in reward; we have focused our attention also on VP and LPB; which are less investigated regions but importantly involved in motivated behaviours, reward and food intake[264,287]. In particular, VP receives projections from ACC and has sparse projections to LC and DR[287]: diet abstinence is able to reduce noradrenergic and serotonergic content of this area, and probably these monoaminergic alterations could be responsible of the link between reward and behavioural impairments in animals subjected to diet withdrawal. On the other hand, enhanced serotonergic transmission is evidenced in LPB of abstinent rats: serotonergic projections from AP and DR in LPB innervate FoxP2 positive neurons that project to VTA, acting on cardiovascular and neuroendocrine functions[288] that can be impacted by this enhanced transmission. Also in this case, PF-3845 treatment in abstinent rats is partially able to restore the alterations detected.

The second part of our study is focused in evaluating the behavioural consequences of prolonged diet abstinence. Our results from OFT, EPM and FST clearly demonstrate that 28 days abstinence from high palatable diet induces anxiety-like behaviour and depressive-like phenotype in rats. Previous experiments preliminary to the present study demonstrated that the consumption of CAFETERIA diet alone was not able to affect emotional behaviour of rats (experiment conducted at the Prof. Carlo Cifani's laboratory at the University of Camerino); so we can speculate that it is the abstinence from, rather than the consumption itself of, high-palatable food the responsible of the behavioural variations.

Likely, these behavioural variations derive from neural impairments in key brain areas linked to anxiety and depression: with this aim we evaluate Pearson's correlative analysis between monoaminergic system and selected behavioural parameters and we discover a significant increment in the number of the correlations between behavioural parameters and 5HT or

5HT turnover in animals abstinent from the diet; suggesting that serotonergic transmission is likely the most involved in the behavioural alterations observed in abstinent animals. In support of this conclusion, we notice important decrease in the number of correlations of 5HT concentration within all the brain areas in rats subjected to the diet withdrawal, in addition to a different pattern when compared to not-abstinent animals. Regarding these variations, PF-3845 administration in abstinent rats is able to reduce and normalize the patterns of correlation between behavioural parameters and 5HT or 5HT turnover and also act on 5HT overall correlations within the different brain areas increasing their number and consequently facilitating serotonergic transmission likely as antidepressant drugs[289].

Serotonergic impairments detected in abstinent rats can be responsible of the depressive-like behaviour that these animals display. Several alterations due to diet withdrawal are detected in DR, the area of synthesis of 5HT: first of all, in abstinent animals we notice a trend toward a decrease of the concentration of 5HT and a trend toward an increase for 5HT turnover. These data could explain why TPH expression is significantly increased in DR of animals subjected to the withdrawal: the brain is trying to compensate the reduced concentration of 5HT by increasing its synthetic enzyme. Moreover, diet withdrawal induces increased TH expression in DR (sign of increased DA projections in this area) and a significant decrease of DA concentration: up to date, the role of dopaminergic neurons in DR has yet to be fully clarified, but it is known that dopaminergic neurons from VTA innervate DR serotonergic neurons that in turn project to dopaminergic neurons of SN[290]. Decreased DA amount in DR may lead to decreased serotonergic neuron activation and consequently, depressive-like behaviours[215] and diminished serotonergic activation of SN which, as said before, can cause reward impairments. 5HT impairments in abstinent rats may also be driven by endocannabinoid system alterations since CB1 activation in this area inhibits 5HT release[291] and these animals display increased CB1 expression; as well as could be caused by FAAH significant decrease since selected genetic deletion of FAAH in DR has been proven to alter serotonergic transmission and emotional behaviour[292]. As a consequence, cafeteria abstinent rats in DR display also variations in the correlation pattern between the proteins of the endocannabinoid system, revealing its impairment in this region. However, depression-like behaviour of abstinent rats depends also on variations in limbic areas, involved in emotionality and mood tone: for example reduced expression of CB2 occurring in the mPFC of animals subjected to the withdrawal has been linked to depressive phenotypes[293].

Moreover, both in PFC and mPFC diet withdrawal causes an increase in the number of correlations between monoamines and proteins of the endocannabinoid system and a variation of the correlation pattern, suggesting once more that cafeteria diet exposure and withdrawal can modify the "normal" functional interactions between the two systems.

On the other hand, PF-3845 treatment in abstinent rats was able to partially restore the alterations caused by diet withdrawal in DR, mPFC and PFC; re-establishing a "normal" correlation pattern between monoamines and endocannabinoid system proteins in DR and decreasing the number of correlations in mPFC and PFC compared to non-treated rats. In addition, PF-3845 administration increases NAPE PLD activity in DR and so, enhances AEA production in this area, 5HT neurons firing activity and 5HT release[254].

Anxiety-like behaviour displayed by abstinent rats can be explained by several variations occurring to noradrenergic transmission and its circuitry. In particular, cafeteria diet abstinence alters the Pearson correlation pattern of NA concentration within all the areas when compared to chow-fed animals, suggesting functional impairments of NA transmission throughout the brain. In addition, abstinent rats display an increment of NA synthesis in LC, due to increased DBH expression, but not an increase of NA concentration in the same area; suggesting that NA is being produced and rapidly metabolized or directly released in sites of projection.

In addition to noradrenergic transmission variations, the anxiety-like behaviour displayed by abstinent rats is compatible with endocannabinoid system alterations detected in AMY: the significant decrement of NAPE-PLD that we observe in this area in abstinent rats can be linked with reduced production of AEA, which in this area is fundamental to regulate stress, anxiety and fear[294], emotional memory and plasticity[295]; moreover similar alterations to MAGL expression in AMY have been linked to anxiety-like behaviour in response to alcohol dependence[296]. In addition, both in LC and AMY we notice a decrease in DAGL alpha expression: variations in 2-AG levels have been implicated in alterations of food intake, inflammation, neuroprotection, stress, anxiety, addiction and reward; furthermore DAGL alpha KO animals present an increased depressive and anxiety behavioural phenotype[297]. Furthermore, cafeteria diet exposure is able to vary the correlations between monoamines and endocannabinoid system in both areas when compared to chow feeding; suggesting functional impairments in these areas.

PF-3845 is able to exert an anxiolytic effect: first of all, it significantly improves the behavioural parameters. In addition, PF-3845 administration in abstinent rats is able to increase the expression of endocannabinoid system proteins that are dampened by diet withdrawal in LC and AMY and increase DBH expression in LC compared to vehicle-treated animals. Most importantly, PF-3845 anxiolytic-like effect in abstinent rats is likely linked to the increased NA concentrations in AMY: NA in AMY is able to activate GABAergic neurons that in turn inhibit glutamatergic projections to ACC, HYPO and PFC responsible of anxious behaviours[298]. Furthermore, PF-3845 administration is able to normalize the correlation pattern between monoamines and cannabinoid system proteins of animals subjected to the withdrawal in LC and facilitate cannabinoid transmission in AMY, as demonstrated by increased correlations between the endocannabinoid system proteins in this area when compared to vehicle administered rats: enhanced cannabinoid transmission in AMY has been suggested as a mechanism of anxiolysis for FAAH inhibitors[252].

Besides LC and AMY, also dorsal and ventral hippocampus are involved in anxiogenic-like response, but no significant variations in monoaminergic systems have been noticed neither due to cafeteria diet exposure, nor due to pharmacological treatment. Another area that in the last years has been linked to the control of anxiety is PAG[299]: we cannot exclude that increased TH and CB1 expression noticed in animals subjected to diet withdrawal may have a role in their behavioural alterations, since HPA axis activation turns into PAG activation[300] and several projections from PAG innervate AMY[299].

As widely explained in the introduction, anxiety and depression can be the consequence of altered HPA axis activity and neuroinflammation. Contradictory studies on the effects of stress on the endocannabinoid system have been conducted and demonstrate that different stressor agents could lead to different variations on endocannabinoids[301]. In our case, diet abstinence causes a decreased activation of the endocannabinoid system in HYPO, as shown by the decrease of correlations in this area in abstinent rats when compared to chow-fed ones; that can be justified considering that chronic stress decreases AEA amount in this area and CB1 expression[266]. Unexpected results came from the analysis of markers of neuroinflammation: even though we observe a trend toward the increase for the concentration of COX-2 in the HYPO of animals abstinent from the diet, suggesting

prostaglandin production and inflammatory process development[157], in almost all the areas we observe a decrease of the expression of GFAP and IBA-1 markers, underlining a reduction of glial and microglial activation. Up to date, several studies have shown that obesity is associated with neuroinflammation[151], but no study so far investigated these markers variations in response to high-fat diet withdrawal. Additionally, both glial and microglial cells have an important role in eliminating additional synapses and data reported by the literature evidence that ablation or manipulation of these cells leads to persistent excess synaptic connections associated with changes in neural circuits and behaviour[302]. We can consider these data consistent with the correlations studies that we have carried out: in particular, Pearson correlations analysis evidence that animals abstinent from the diet present different patterns of correlations both for the single protein or monoamine across the different brain regions and for monoamine and cannabinoid correlations within the same region when compared to chow-fed ones; suggesting that diet withdrawal can impact on brain functional connectivity so far.

Once more, PF-3845 administration contributes to normalizing the variations caused by diet withdrawal: in particular, it is able to significantly dampen COX-2 and DAGL alpha expression in HYPO; reducing prostaglandin production and the synthesis of 2-AG (normally increased by stress in this area[301]). Generally, PF-3845 administration is able to increase GFAP and IBA-1 expression in all the areas analysed, to levels comparable to the ones of chow-fed animals. On the other hand, PF-3845 administration did not normalize all the patterns of correlation of proteins across the different brain regions and of monoamines and proteins within the same brain region: generally animals subjected to diet withdrawal and treated with PF-3845 display a pattern of correlation which is different both from vehicle treated animals subjected to diet abstinence or fed with chow; however we cannot exclude that these variations are the ones responsible for PF-3845 anti-depressant and anxiolytic mechanism of action.

To sum up, we can state that CAFETERIA diet exposure and subsequent abstinence in rodents is able to affect the reward system by reducing total dopaminergic activity, altering the VTA excitability and the activation of SN. At the same time, key brain areas as ACC and DLS appear to be hypo activated and to have less dopaminergic projections. Diet abstinence

causes several alterations of monoaminergic and endocannabinoid systems, compatible with addictive behaviour and reward impairments. Abstinent rats display anxiety-like behaviour and a depressive-like phenotype (compatible with decreased serotonergic activity); justified by several impairments in DR, PFC and mPFC monoaminergic and cannabinoid systems. Abstinence impacts also noradrenergic transmission, by increasing DBH activity in LC and causes several alterations in AMY compatible with anxiety and stress. Furthermore, abstinent rats display peculiar neuroinflammation profile, with a reduced glial and microglial expression.

PF-3845 administration in abstinent rats can be considered advantageous: pharmacological treatment is effective in increasing total dopaminergic activity, stimulating the activation of SN through various mechanisms and restoring the alterations in endocannabinoid system detected in reward areas. In addition, PF-3845 exerts anti-depressive and anxiolytic effect, promoting serotonergic activity and re-establishing the alterations detected in LC and AMY. Pharmacological treatment results effective also in decreasing prostaglandin production in the HYPO and increasing microglial and glial population altered by diet abstinence.

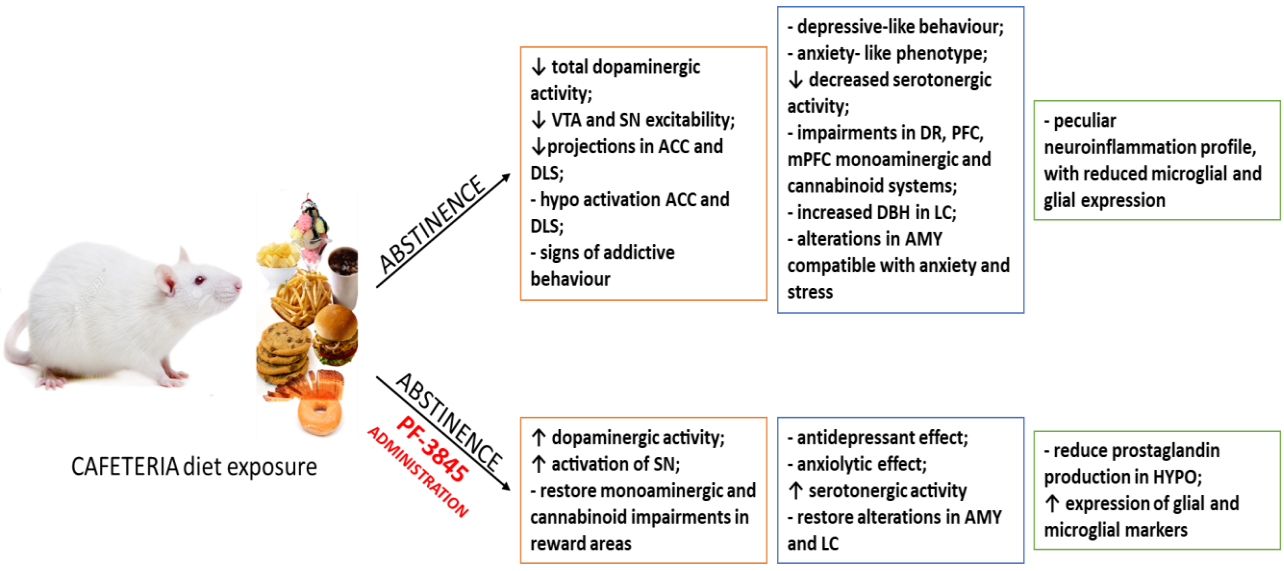

Fig.1: Summary of the main points of the discussion.

However, several limitations may be found to our study: first of all, only male rats were used in the experiment, in order to simplify experimental procedures and not consider hormonal variations typical of female exemplars. Eating behaviour is highly influenced by menstrual cycle and female hormones, so we cannot exclude that our findings could vary in a female population. Moreover, even if no behavioural variations were noticed after 40 days of CAFETERIA diet exposure, we have not evaluated the impact of high-palatable food exposure per se, that can cause alterations in the monoamine concentration, expression of all the proteins analysed for each brain area and their correlations. Other limit can be considered the use of CO_2 for the sacrifice: this type of euthanasia can alter stress hormone response and influence HPA axis activation, cause momentary pain in the rat and in some cases excitation and agitation, which can impact on the studies carried in the brain.

At the same time, further analysis could be run in our model: first of all, it would be interesting to evaluate endocannabinoids and acylethanolamides concentrations in all the areas analysed and correlate them with monoamines and expression of proteins partaking to the endocannabinoid system both in animals abstinent from the diet and subjected to the pharmacological treatment. Moreover, a deeper investigation of the expression of the receptors of DA, NA and 5HT could help in elucidating further aspects of reward and behavioural alterations in animals abstinent from the diet and of PF-3845 mechanism of action. Further study on neuroinflammatory state could be run, such as analysis of cytokines, interleukines and stress hormones. Other interesting studies could focus on synaptic density, synaptogenesis and dendritic ramification; that can be impacted by obesity and acylethanolamides manipulation as well.

CHAPTER 5 – CONCLUSIONS

Obesity is a pandemic disease[2]. Various reasons caused the spread diffusion of the pathology worldwide, and among them, overeating and sedentary lifestyle are for sure two important factors[303]. Several studies aim to investigate what environmental, genetic, neurological impairments are at the basis of obesity development[5], and in the last years accumulating evidence suggested the existence of a type of hedonic obesity, caused by relevant variations to reward associated to food cues[16]. At the same time, several observations suggests that the pathology can be considered as an addiction to food that the individual struggles in controlling, likely what happens with drug addiction[36]. In fact, the majority of times, food is consumed by the individual to overcome negative states deriving from the mind and overeating becomes the answer to everyday life problems[8]. Lots of pathologies have been associated with obesity, but up to date, less importance has been given to mental health diseases, whose link with obesity is poorly defined: obesity causes a chronic state of inflammation[151], neuroadaptive changes that involve several neural circuits and can lead to the development of depression and anxiety[142]. Investigating the role of obesity in these pathologies is fundamental: no effective one-size-fit-all treatment is available up to date, and there is the necessity of finding new pharmacological targets that can be addressed to treat it[15].

With our study, we demonstrate that exposure and long-term abstinence from a palatable cafeteria diet provokes anxiety-like behaviour and depressive-like phenotype in rats. The behavioural alterations are accompanied by several neuroadaptive changes in brain monoaminergic, endocannabinoid system and their functional connections in key brain areas linked with reward, anxiety and depression, despite the fact that at the end of abstinence rats have reduced their body weight. Moreover, our results demonstrate that the pharmacological manipulation of endogenous acylethanolamides through FAAH inhibition is able to exert an antidepressant and anxiolytic effect, modulating both the monoaminergic and the endocannabinoid system. Other important actions attributed to FAAH inhibition are modulation of

reward system, dampening of addition, neuroprotection and anti-inflammatory properties[182,304].

All these evidences once more confirm the endogenous role of acylethanolamides (AEA, PEA and OEA) not only in the control of food intake[255], but also in mood alterations and neuroinflammation; thus sustaining our thesis that the endocannabinoid system and its modulation can be a new target for the treatment of obesity-related comorbidities, moving the attention from “classical” CB1 antagonists, such as Rimonabant (characterized by important adverse reaction) to FAAH inhibitors.

Up to date, several clinical studies have investigated the use of FAAH inhibitors to treat different conditions: anorexia, neuropathic pain, multiple sclerosis. In the beginning, neurological side effects (cognition, motor disfunctions, psychosis) were noticed only in long-term treatment, but lots of trials were temporarily suspended after the death of a phase I healthy volunteer administered with the FAAH inhibitor BIA 10-2474. This tragedy was completely unexpected and was demonstrated to be caused by off-target effects of BIA 10-2474 and/or its metabolites[305].

However, we sustain that our study is important in evidencing that palatable food exposure and subsequent abstinence can cause neuroadaptive changes that turn into the development of depression and anxiety, raising the importance that these comorbidities and their treatment have in obesity and palatable food consumption. Most importantly we demonstrate the pivotal role of acylethanolamides and endocannabinoid system in obesity and related comorbidities; sustaining that their modulation could be used as target for new pharmacological strategies addressed to an effective treatment of the pathologies.

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ABBREVIATION LIST

2-AG	2 acyl-glycerol
5HIAA	5-hydroxy indoleacetic acid
5HT	serotonin
5HTR	serotonin receptor
5HTR	serotonin receptor
AADC	L-aromatic amino acid decarboxylase
ABHD4	alpha/beta hydrolase domain 4
ACC	nucleus accumbens
AEA	anandamide
AgRP	Agouti related peptide
ALDH	aldehyde dehydrogenase
AMY	amygdala
ARC	arcuate
BBB	blood brain barrier
BDNF	brain derived neurotrophic factor
BLA	basolateral amygdala
BMI	body mass index
BNST	bed nucleus of stria terminalis
CAF	cafeteria
CAMKII	calcium calmodulin dependent protein kinase II
cAMP	cyclic adenosine mono phosphate
CART	cocaine and amphetamine related transcript
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
CCK	cholecystokinin
CCK1	cholecystokinin receptor 1
CEA	central amygdala
CNS	central nervous system
COMT	catecholamine methyl transpherase
COX-2	cyclooxygenase 2
CREB	cAMP response element-binding protein
CRF	corticotropin releasing factor
CRH	corticotropin-releasing hormone
CYP	cytochrome P450
D1	dopamine receptor 1
D2	dopamine receptor 2
D3	dopamine receptor 3
D4	dopamine receptor 4
D5	dopamine receptor 5
DA	dopamine
DAG	diacyl-glycerol
DAGL	diacil glycerol lipase
DARPP-32	dopamine and cAMP regulated phosphoprotein 32-kDa
DAT	dopamine transporter
DAT	dopamine transporter

DBH	dopamine beta hydroxylase
DDC	dopamine decarboxylase
dHIPPO	dorsal hippocampus
DLS	dorsolateral striatum
DMN	dorsomedial nucleus
DMS V	diagnostic and statistical manual of mental disorders
DOPAC	3,4-diidroxyphenilacetic acid
DOPAL	3,4-diidroxyphenylacetaldehyde
DR	dorsal raphe
EE	emotional eating
ENS	enteric nervous system
EPM	elevated plus maze
ERK	extracellular regulated kinase
FA	food addiction
FAAH	fatty acid amine hydrolase
fMRI	functional magnetic resonance imaging
FST	forced swimming test
GABA	gamma aminobutyric acid
GFAP	glial fibrillar acidic protein
GHS-R	growth hormone secretagogue receptor
GI	gastrointestinal tract
GIRKs	G protein inwardly rectifying potassium channels
GLP-1	glucagon like peptide 1
GLP-1R	glucagon-like peptide receptor type 1
GRK	G protein receptor kinase
H1R	histamine receptor 1
HPA	hypothalamus-pituitary axis
HPLC	high performance liquid chromatography
HVA	homovanillic acid
HYPO	hypothalamus
IL	interleukin
IP3	inositol triphosphate
LC	locus coeruleus
L-DOPA	levodopa
LIF	leukaemia inhibitory factor
LPB	lateral parabrachial
MAGL	mono acyl glycerol lipase
MAPK	mitogen-activated protein kinase
ME	median eminence
MOPEG	3-Methoxy-4-hydroxyphenyl glycol
mPFC	medial prefrontal cortex
NA	noradrenaline
NAAA	N-acylethanolamine-hydrolysing acid aminase
NAPE-PLD	N-acyl phosphatidylethanolamide lipase D
NArPE	N-arachidonoyl-phosphatidyl ethanolamides
NET	norepinephrine transporter
NPY	neuropeptide Y
NST	nucleus of solitary tract

OD	optical density
OEA	oleoylethanolamide
OFT	open field test
OXM	oxyntomodulin
PAG	periaqueductal grey
PEA	palmitoilethanolamide
PFA	paraformaldehyde
PFC	prefrontal cortex
PKA	protein kinase A
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
POMC	pro-opiomelanocortin
PP	pancreatic peptide
PP1	protein phosphatase 1
PP2B	protein phosphatase 2B
PPARα	peroxisome proliferator-activating receptor alpha
PVN	paraventricular nucleus
PYY	peptide YY
ROS	reactive oxygen species
SERT	serotonin transporter
SN	substantia nigra
STEP	striatal enriched protein phosphatase
TH	tyrosine hydroxylase
TNF-α	tumoral necrosis factor alpha
TPH	tryptophane hydroxylase
TRPV1	transient potential vanilloid receptor 1
VEH	vehicle
vHIPPO	ventral hippocampus
VMAT	vesicular monoamine transporter
VPL	ventral pallidum
VTA	ventral tegmental area
WB	western blot
Y2R	Y2 receptor
YFAS	Yale food addictionScale
α-MSH	α- melanocyte stimulating hormone

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