Facultad
de Psicología

UNED

UNIVERSITÀ DEGLI STUDI DI TORINO

DIPARTIMENTO DI: NEUROSCIENZE

UNIVERSIDAD NACIONAL DE EDUCACIÓN A DISTANCIA

DIPARTIMENTO DI: PSICOLOGIA

DOCTORAL RESEARCH IN NEUROSCIENCE AND PSYCHOLOGY

CYCLE: **XXXIII**

TITLE OF THESIS: **'THE ACTIVITY-BASED ANOREXIA MODEL: BEHAVIORAL AND MORPHOLOGICAL STUDIES'**

CANDIDATE: **GODSTIME STEPHEN KOJO MORGAN**

TUTORS: **PROF. STEFANO GOTTI**

PROF.SSA MARIA ELENA PINOS SANCHEZ

DOCTORAL RESEARCH COORDINATORS: PROF. ANDREA CALVO

PROF. RICARDO PELLÓN SUÁREZ DE PUGA

ACADEMIC YEARS: **2017/2021**

AFFILIATED SCIENTIFIC DISCIPLINARY SECTOR: BIO/16; PSI/01

TABLE OF CONTENTS

ABBREVIATIONS

5-HIAA: 5-hydroxyindoleacetic acid

5-HT: serotonin

ABA: activity-based anorexia

AMPA: α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate

AN: anorexia nervosa

ARC: arcuate nucleus

ASST: attentional set-shifting test

BED: binge eatingdisorder

BN: bulimia nervosa

CeA: central nucleus of the amygdala

CON: control

CRH: corticotropin-releasing hormone

CSF: cerebrospinal fluid

DA: dopamine

DMN: dorsomedial nucleus

DNA: deoxyribonucleic acid

DR: dorsal raphe

DRN: dorsal raphe nuclei

DSM: diagnostic and statistical manual of disorders

ED: eating disorders

EPM: Elevated Plus maze

F: female

FAA: food-anticipatory activity

fMRI: functional magnetic resonance imaging

FS: foot shock stress

GABA: gamma-aminobutyric acid

HPA: hypothalamic-pituitary-adrenal axis

L-DOPA: levodopa

M: male

MR: median raphe

MS: maternal separation

NA: nocturnal activity

NAc: nucleus accumbens

NE: norepinephrine

NH: non-handled

NMDA: N-methyl-D-aspartate

NOR: novel object recognition task

OF: open field

PET: positron emission tomography

Pmch: pro-melanin-concentrating hormone

PND: postnatal day

PPA: postprandial activity

PS: psychological stress

PVN: paraventricular nucleus

ROI: region of interest

SBA: separation based anorexia

SHRP: stress hyporesponsive period

SNK: Student-Newman-Keuls

SNpc: substantia nigra pars compacta

SPM2: statistical parametric mapping

SSRIs: serotonin-selective reuptake inhibitors

TH: tyrosine hydroxylase

TLRs: toll-like receptors

VTA: ventral tegmental area

CHAPTER 1: GENERAL INTRODUCTION

1.1 EATING DISORDERS

Eating disorders (ED) refer to the disturbances in eating habits characterized by excessive food intake or restriction resulting in energy imbalance. They are often associated with high comorbidity and chronic severe health consequences, due to resistance to conventional treatments by some subsets (Abbate-Daga et al., 2013; Novelle & Dieguez, 2018). Their cause is elusive, with social, psychological, and biological processes all seeming to play a significant part (Fairburn & Patel, 2014). Although the prevalence of ED has remained stable, the high mortality rate and their association with other psychiatric disorders, have generated great public interest. Also, it has posed a challenge to clinicians and a concern to researchers in an attempt to clarify the genetic, neurochemical, and physiological substrates implicated in their development (Fairburn & Patel, 2014).

Previously, in the Diagnostic and Statistical Manual of Disorders, Fourth Edition (DSM-4), ED were classified as anorexia nervosa (AN), bulimia nervosa (BN) and atypical eating disorders (Marzilli et al., 2018). Currently, in the (DSM-5), changes have been made in the classification of ED: AN, BN, and binge eating disorder (BED). The category "Eating Disorders Not Otherwise Specified" has been renamed as "Unspecified Feeding or Eating Disorders (Knoll et al., 2014).

AN is primarily denoted by extreme low body weight and an intense fear of its increase, distorted body image, and a global endocrine dysregulation predominantly in women and adolescent girls (DSM-5) (Harrington et al., 2015; Schorr & Miller 2017). It has a lifetime prevalence of 0.3 - 0.9 % with onset predominantly during the early adolescence stage; however, prepubescent onset and diagnosis in women in midlife and early adulthood are also common (Bulik, Yilmaz & Hardaway, 2015). Due to the presence of a regular menstrual cycle in some women with low body weight and presenting most psychiatric features of AN, amenorrhea is no more included in the AN diagnosis criteria (DSM-5; Harrington et al., 2015; Schorr & Miller 2017).

BN is characterized by repeated binge eating, followed by behaviors to counteract it, such as vomiting, excessive exercise, taking laxatives, or using diuretics to prevent weight gain (DSM-5; Harrington et al., 2015; Skunde et al., 2016). The lifetime prevalence of BN is 0.8 – 2.9 % and predominant in females compared to males. The onset is normally later than in AN but can also occur during the adolescent stage (Bulik, Yilmaz & Hardaway, 2015). Individuals with BN usually have normal weight or overweight and unlike in the DSM-IV, a purging episode of at least twice a week for six months has been reduced to once a week for three months in the DSM-5 for BN diagnosis (Harrington et al., 2015; Bulik, Yilmaz & Hardaway, 2015). Due to the frequent vomiting

in BN patients, there is usually the presence of dental enamel erosions and gum diseases (Harrington et al., 2015).

BED, introduced in May 2013 as a separate type of ED (DSM-5), is noted as one of the most serious types of ED among the youths, early to mid-'20s (DSM-5) (Bulik, Yilmaz & Hardaway, 2015; Nicholls et al., 2016; Marzilli et al., 2018), with a lifetime prevalence estimated between 2 and 3.5 % (Bulik, Yilmaz & Hardaway, 2015). It is characterized by frequent intake of a large amount of food without compensatory behaviors as observed in BN (Marzilli et al., 2018). Subjects usually have feelings of low self-esteem, loss of control, and marked distress (Hilbert et al., 2011; Marzilli et al., 2018).

1.2 ANOREXIA NERVOSA

Amongst all the eating disorders, AN is known to be the most severe type recording the highest mortality rate of any psychiatric disorder (Franko et al., 2013). It is a complex and multifactorial disorder resulting from environmental stressors as well as genetic factors in which, according to twin studies, heritability estimates in the range of 48 – 74 % have been indicated (Casper et al., 2008; Ribases et al., 2013; Madra & Zeltser, 2016: Duncan et al., 2017). A recent work that analysed a theoretical ED model argued that patients with AN present a profound and significant alteration in a wide network of brain areas related to the development of the integrative function of the self (Amianto et al., 2016). In addition, authors also mention a strong association between a deficit in the self with attachment insecurity, sociocultural factors, traumatic experiences and genetic vulnerabilities of being underweight (Amianto et al., 2016).

Increased activity of the hypothalamic-pituitary-adrenal (HPA) axis is among the primary indicators of anorexia nervosa (Hancock & Grant, 2009). In animal models experimental manipulations, such as early maternal separation, premature weaning, and social isolation, are used to analyse some aspects of the human AN disorder. These manipulations result in the hyperactivity of the HPA axis in these models, hence, confirming the alteration in this axis as a risk factor for the development of anorexia (Hancock & Grant, 2009).

The severe malnourishment observed in AN patients results in endocrinological and cardiological dysfunctions as well as numerous abnormalities within the digestive, skeletal, and reproductive systems (Grzelak et al., 2017).

It is worthwhile also to mention that, differences exist amongst AN patients and these differences stem from the subtypes of AN (restrictive and bulimic-purging type), the course and stage of the

disorder, attitude towards treatment, and how some patients perceive consequences of the disorder (Abbate-Daga et al., 2013; Grzelak et al., 2017).

A well-marked treatment for AN condition is still not known; nevertheless, significant advances and understanding of the underlying biology in this condition have been elucidated thanks to basic research in several animal models (van Elburg et al., 2007; Bulik, Yilmaz & Hardaway, 2015). This milestone is essential because it has contributed to the development of more satisfactory and effective pharmacological treatment to minimize the low quality of life, high treatment cost, and high mortality rate observed in the AN patients (van Leeuwen et al., 1997; Welch et al., 2018).

1.3 ANIMAL MODELS USED IN THE STUDY OF ANOREXIA NERVOSA

During the last years, several animal models have been devised to simulate replicable biological correlates and underline the pathophysiological condition in AN patients (Table 1) (van Leeuwen et al., 1997). Even though not always feasible to satisfy, an optimal AN model should have some essential characteristics (Zgheib et al., 2014). In particular, the animal model should be developed in young animals (preferably females) and be of enough duration to allow for long-term adaptations as well as the development of changes/alterations in tissues. Moreover, the experimental model should reflect original features seen in AN patients and include, if possible, a recovery phase (Zgheib et al., 2014).

1.3.1 Genetic model

Models with spontaneous mutations and genetically engineered animals are used in the study of AN disorder to analyse the involvement of several genes in feeding and metabolism (Alliot et al., 2002; Nilsson et al., 2013; Bulik, Yilmaz & Hardaway, 2015). The pro-melanin-concentrating hormone gene, which encodes the neuropeptide melanin-concentrating hormone, has been elucidated in a kind of knockout mice, the *Pmch-/- mice* (Bulik, Yilmaz & Hardaway, 2015). It was observed that the absence of the gene's product causes hypophagia and lean phenotypes, which resulted in reduced body weight and food intake and upon intracerebroventricular injection of the peptide in the knockout mice, food intake was increased (see Table 1) (Bulik, Yilmaz & Hardaway, 2015). Another spontaneous mutation model, the *Lou/C rat*, has typical symptoms observed in anorexia including an up-regulation in expression of AgRP and NPY and a downregulation in ghrelin and leptin levels (see Table 1) (Alliot et al., 2002). Although these kinds of models are appropriate to study some characteristics of anorexia, there are some limitations: for instance, in a

model with spontaneous mutations such as the *anx/anx mice*, animals do not reach puberty due to their short life span (about 3 weeks after birth) (see Table 1) (Maltais et al., 1984; Lindfors et al., 2011); however, it has been validated as an excellent model of anorexia-cachexia syndrome characterized by an inflammatory response (Lindfors et al., 2011).

The monoamines, especially dopamine and serotonin alterations, have been implicated in AN (Szczypka et al., 2001). A dopamine and norepinephrine deficient mice developed by targeting the tyrosine hydroxylase coding sequence in mice, *DA-/- mice*, was used to analyse the effect of dopamine on activity and feeding behaviors (Szczypka et al., 2001). The mice usually grow about a week but gradually become hypophagic and undersized due to inadequate feeding and die by four weeks of age. L-DOPA daily treatment was observed to increase the locomotor and feeding behavior of these mice however, these behaviors decline after few hours once the DA is metabolized (see Table 1) (Zhou & Palmiter, 1995; Szczypka et al., 2001). Authors reported a regular neural development in the *DA-/- mice*, indicated by the observation of DA neurons and their terminals after an immunostaining technique with an antibody against the enzyme that converts L-DOPA to DA; however, an aberrant DA signaling in the mice was observed (Zhou & Palmiter, 1995; Szczypka et al., 2001). This model, as much as being efficient in the study of some underlying mechanisms in AN, is limited because L-DOPA administration has a time window (Szczypka et al., 2001).

Mechanisms linked to processes regulating feeding and metabolism can be studied with the genetically engineered animal models (Alliot et al., 2002), but these models do not allow the evolution of the anorexic phenotype (Lindfors et al., 2011); hence, environmental models able to cover a broader aspect of the pathology are recommended.

1.3.2 Environmental model

Under minimal presentation of a stressor, the brain correctly copes with the new condition by activating the autonomic nervous system and the HPA axis (Quaedflieg et al., 2013). The former acts to increase alertness and arousal by inducing the release of adrenaline and noradrenaline, whereas the latter activates memory processes by inducing the release of glucocorticoids (Quaedflieg et al., 2013). When the effect of a stress is seen shortly after induction, it is termed immediate, whereas, when a time-period is allowed between the stress and its effects it is termed historic stress (Corwin & Buda-Levin, 2004). The latter is suitable in the analysis of vulnerabilities provoked by certain early life events, as seen in several eating disorders (Rorty et

al., 1994: Corwin & Buda-Levin, 2004). For instance, in the work of Rorty et al., the authors reported that women with bulimia nervosa have generally experienced elevated levels of trauma during the childhood period (Rorty et al., 1994).

Models based on stress, either acute or chronic, use procedures such as, but not limited to, tail pinching, cold swim, exercise wheels, direct brain stimulation, maternal separations, and physical isolation (van Leeuwen et al., 2011). Acute stress is reported to be associated with increased activation of neurons involved in stressful stimuli processes and responses, whereas those induced with chronic stress usually have lowered effects due to adaptation (Chagra et al., 2011).

1.3.2.1 Separation induced weight loss model

This model has been elucidated partially in several articles (Hao et al., 2001; van Leeuwen et al., 2011). Seventeen-weeks old female Sabra mice were housed in a cage fitted with Perspex partitions in order that they could see but not touch or smell each other and transferred to the same cage during the feeding time (Casper et al., 2008). In the original protocol, two main procedures were employed for the food restriction, aside from the primary stressor being separation. Groups of animals received either a percentage of the calculated daily nutritional requirement or underwent a 1 hr per day feeding schedule with a 24-hr water supply (Hao et al., 2001; van Leeuwen et al., 2011; Zgheib et al., 2014). The separated groups were noted to have a restricted food intake and an increase in energy expenditure (van Leeuwen et al., 2011). The separation, according to Hao and colleagues (2001), can induce physiological consequences such as decreased bodyweight, spontaneous T-maze alternation, depletion of both norepinephrine (NE) and dopamine (DA) as observed in AN patients (see Table 1). The administration of tyrosine reversed the decreased NE and DA concentration in the hippocampal region through an intraperitoneal injection leading to an improvement in the behavioral analysis (Hao et al., 2001; Zgheib et al., 2014).

1.3.2.2 Separation based anorexia (SBA) model

A group of researchers headed by Zgheib in 2014 coined the name SBA as a model to study anorexia (Zgheib et al., 2014). The model evolved from the 'separation induced weight loss model' with the inclusion of a feeding scheme where food is gradually reduced from 6h to 2h a day along with the protocol. It was used in seven-week-old female C57BL/6J mice, and it encompassed a time-restricted feeding and stress induced by separation. The combination of both time-restricted

feeding and separation contributed to severe body weight and fat mass decline; however, food consumption was not affected, thus, indicating a modified energetic balance (Table 1) (Zgheib et al., 2014).

Authors underlined essential aspects of this model mentioning that the SBA can induce a fast bodyweight decline of at least 25% of the initial weight and the bodyweight reduced could be maintained for a period, 8-10 weeks, permitting a long-term protocol of recovery. Moreover, animals under this model exhibited hypogonadism and alteration of vital endocrine parameters (e.g., hypoleptinemia) (Table 1) (Zgheib et al., 2014).

Taking together, the SBA model is confirmed as a valuable tool to analyse some of the primary physiological alterations observed in AN patients.

1.3.2.3 Activity based anorexia (ABA) model

ABA model was introduced by Routtenberg and Kuznesov (1967) and described as the first principal model for self-starvation in animals (Routtenberg & Kuznesof, 1967; Boakes RA, 2007; van Leeuwen et al., 2011). In the original ABA model, adult male albino rats were exposed to a restricted feeding scheme, 1 hr per day, free access to water and unlimited access to a running wheel (Table 1) (Routtenberg & Kuznesof, 1967; Boakes RA, 2007; van Leeuwen et al., 2011; Welch et al., 2018). This induced a steady weight loss over 14 days (Routtenberg & Kuznesof, 1967; Boakes RA, 2007) as rodents starved themselves to death and the weight loss could only be stopped once the rodents were removed from the ABA protocol (Routtenberg & Kuznesof, 1967). Paradoxically, the loss of the weight was accompanied by an intense activity on the running wheel. The ABA model is demonstrated to have a significant impact on energy metabolism, reward circuitry, and bone physiology, alterations present in about 80% of AN patients (Table 1) (Hebebrand et al., 2003; Zgheib et al., 2014).

The extent of weight loss associated with the wheel activity is influenced by many factors (Gutierrez et al., 2002). One primary factor is age: the effect is much stronger in younger rodents (Woods & Routtenberg, 1971; Boakes RA, 2007); rodents that received enough maternal interaction were less affected when subjected to the protocol (Carrera et al., 2006; Hancock & Grant, 2009); a more extended feeding period or increase in the number of feeding times at the same total time duration as a single period indicated a slower weight loss and less likely for self-starvation to occur (Carrera et al., 2006; Boakes RA, 2007). Reducing the period spent on the

running wheel also allowed to regulate the effect (Hancock & Grant, 2009). Thermogenic homeostasis has been debated to be one of the reasons for the excessive activity due to the assertion that rodents (especially rats) prefer a warmer body temperature when there is a progressive loss in their body weight (Sakurada et al., 2000; Boakes RA, 2007).

1.3.2.4 Mild-stress ABA protocol

Due to the rapid weight loss and intense running induced by the ABA original protocol, most features cannot be studied since the rodents either die early or are removed from the paradigm in order to survive (Routtenberg & Kuznesof, 1967). A mild-stress ABA protocol has been observed to produce suppression of food intake and reduction of body weight characteristic of the original ABA protocol but to a lesser extent than that produced by unlimited wheel access (Hancock & Grant, 2009; Farinetti el al., 2020). This reduced progression of the disorder, primarily from the running-wheel access modulation, is essential to allow time for the development of alterations characteristic of anorexia since these alterations are mostly present at the later stages of the disorder (Zgheib et al., 2014).

In the work of Hancock and Grant it was stated that *"even under the relatively mild conditions of 2-hr running wheel access in combinations with a 1-hr restricted feeding schedule, our animals demonstrated features characteristic of ABA"* (Table 1) (Hancock & Grant, 2009).

1.3.3 Distinctiveness of the ABA protocol

ABA is a bio-behavioural phenomenon described in rodents that can simulate features or symptoms of the human AN disorder including the restricted food intake in the presence of hunger, weight loss, drive for activity, and the physiological consequences of under-nutrition; however, it does not explore the socio-cultural factors of the disorder (Casper et al., 2008; Hancock & Grant, 2009; Chowdhury et al., 2015). Notwithstanding, ABA models share many behavioral and physiological parameters similar to patients with AN (Lamanna et al., 2019). An important aspect of the model is the interrelationship between time-limited food availability and running wheel activity: separately, no significant impact is observed (Foldi et al., 2017) especially on body weight because when only food-restricted subjects quickly enhance food intake to increase their weight (Foldi et al., 2020). The synergy between these two factors of the ABA model allow investigation of the effect of stress in order to bring to light some aspects of AN relapse (Chowdhury et al., 2015).

The body mass index, measured as the ratio between body mass (kg) and the square of height (m), is one of the main factors considered in treatment efficacy in AN (Wu et al., 2014, Carter et al., 2004). In fact, previous studies geared towards the analysis of relapse in AN primarily considered body weight loss (Carter et al., 2004). Similarly, in the ABA protocol, the subject is removed from the protocol after attaining a weight loss of 25% with respect to baseline body weight (Routtenberg & Kuznesof, 1967).

A salient feature observed in patients with AN is the poor cognitive flexibility associated with excessive cognitive control (Lamanna et al., 2019). Cognitive inflexibility, responsible for the appetitive control inhibition and a persistent drive for weight loss irrespective of the negative effect of starvation, is a parameter involved in the development of the AN pathology in the acute phase and contributes to the difficulty in disease treatment (Allen et al., 2017, Lamanna et al., 2019; Foldi et al., 2020). Interestingly, this deficit in AN patients has been evaluated in an ABA model using the attentional set-shifting test (ASST) (Allen et al., 2017). This test allows for the analysis of performance in discriminative learning, reversal learning and set-shifting. Compared with weight loss-paired control group, authors observed that ABA-induced weight loss (about 20% loss) had a significant alteration in reversal learning, a deficit reversed following weight restoration. Thus, the utility of the ABA model could help to study the cortical dysfunction in such deficits (Allen et al., 2017).

In relation to memory, the hippocampus is very instrumental due to its function of transmitting memory content from short to long-term (Schalla et al., 2019). Analysis of this structure in AN patients revealed a markedly reduced volume (Connan et al., 2006; Lamanna et al., 2019). This defect suggests memory impairment even though no significant reduction in memory performance was found in the patients. In comparing two groups of ABA rats: one group was sacrificed directly after reaching a 25% body weight loss (acute starvation) and other group was kept for an additional two weeks receiving adjusted food intake to maintain the 25% body weight loss (chronic starvation), authors observed a reduction in the hippocampal volume as well as the regulator of the hippocampal function, 17-beta oestradiol during the chronic starvation period The reduction was associated with a profound memory impairment during a novel object recognition (NOR) task (Paulukat et al., 2016; Lamanna et al., 2019). A Barnes maze test showed that the impairment was related to contextual but not spatial memory (Boersma et al., 2016). The changes related to the hippocampus could in part, be associated with altered production of molecules such

as cortisol (in humans) and corticosterone (in animals), a hormone known to endanger neurons of the hippocampus (Connan et al., 2006).

Again, the ABA model allows for the monitoring of cerebral changes. Investigators doing a study in adolescent female Wistar rats observed changes in brain volumes, especially in the cerebral cortex and corpus callosum in a chronic starvation condition. The reduced volume was associated with reduced astrocyte number (Frintrop et al., 2018). Among their many functions, astrocytes are implicated in supplying energy to neurons due to the limited capacity of neurons to store energy (Bélanger et a., 2011). Their reduced number, as explained by the authors, could compromise the energy metabolism of neurons, hence, impairment in neuronal functioning (Frintrop et al., 2018). Again, using statistical parametric mapping (SPM2) strategy to analyse regional brain metabolic changes in ABA models, authors confirmed a decreased metabolic activity in the left rhinal and bilateral insula cortex and bilateral ventral striatum (van Kuyck et al., 2007). ABA rats according to the authors, showed higher metabolic activity in areas such as mediodorsal thalamus, ventral pontine nucleus and cerebellum, concluding that metabolic changes in brain areas in the ABA rats correlate with weight loss and disease status (van Kuyck et al., 2007). Interestingly, the role of the insula has been investigated also in humans affected by AN (Nunn et al., 2008). Nunn et al. using a predominance model, observed that individuals susceptibility to developing AN stems from an impairment in the neural networks converging upon the insular cortex, a condition which impeded the correct integration of visual and body perceptions with emotions and the inhibition of higher cognitive processes (Nunn et al., 2008). Further functional magnetic resonance imaging (fMRI) studies have been performed comparing recovered AN with ill AN women (Frank et al., 2016; Oberndorfer et al., 2013). These studies found only a heightened activation in the posterior and anterior insula in recovered subjects but, in addition to the higher insular response, alterations in other brain regions especially areas associated with dopaminergic function were observed in the ill AN individuals (Frank et al., 2016; Oberndorfer et al., 2013). Suggestively, the increased posterior insular response could be a premorbid condition and able to persist even after recovery (Frank et al., 2016; Oberndorfer et al., 2013).

Currently, conditions such as starvation-induced immunodeficiency and atrophy of the spleen and thymus typically observed in the ABA animal models have not been found in individuals with AN; on the contrary, some authors have reported an increased immune function in the human AN patients (Casper et al., 2008; Chowdhury et al., 2015; Armstrong-Esther et al., 1978). Toll-like receptors (TLRs) are proteins that have a key regulation in innate immune response to bacteria

and also involved in the control of food intake (Belmonte et al., 2016). Using the ABA mice model, Belmonte et al., 2016, observed an upregulation of the TLR4, a condition which contributed to the regulation of intestinal inflammatory response and limitation of bacterial translocation. Authors suggested that TLR4 has a protective role due to the high mortality rate that was evident in TRL4 deficient ABA mice (Belmonte et al., 2016).

Evidentially, irrespective of the limitation of the model in mimicking the psychological aspect of AN, studies employing the ABA protocol have contributed immensely to the establishment of a biologically- based causative model (O'Hara et al., 2015).

Table 1: Summary of animal models with observed alterations in the study of AN

ANIMAL MODELS	STRAIN USED	SEX	FEATURES	OBSERVED ALTERATIONS	REFERENCES
Genetically modified	*Pmch$^{-/-}$ mice* (pups)	n.m.		↓body adiposity ↓ food intake ↓body weight	Bulik, Yilmaz & Hardaway (2015)
	DA$^{-/-}$ mice (pups)	n.m.		↓locomotion ↓food intake Impaired DA signaling	Zhou & Palmiter (1995) Szczypka et al. (2001)
	anx/anx mice (pups)	n.m.	Short life span Anorexia-cachexia model	↓body weight ↓ food intake Head weaving Body tremors Uncoordinated gait ↑serotonin	Maltais et al. (1984) Lindfors et al. (2011) Bulik, Yilmaz & Hardaway (2015)
	Albino *Lou/C* rat (pups)	n.m.		↓ghrelin ↓leptin ↑AgRP ↑NPY	Alliot e al. (2002)
Separation induced weight loss	Sabra mice (17-weeks old)	Females	Separation as stressor 1-hr feeding time or ↓% of food	↓food intake ↓body weight ↓NE and DA ↑energy expenditure Altered T-maze test	van Leeuwen et al. (1997) Hao et al. (2001) Zgheib et al. (2014)
Separation based anorexia (SBA)	C57BL/6J mice (7-weeks old)	Females	Reduced feeding time from 6 - 2 hrs Recovery period	↓body weight ↓fat mass Hypogonadism Hypoleptinemia	Zgheib et al. (2014)
Activity-based anorexia (ABA)	Albino rats (adult) C57BL/6 mice (8-weeks old) Sprague-Dawley rats (8-weeks old)	Males Males and females Males and females	1-hr feeding period 23-hr/day wheel access Effect stronger in younger female models Greater susceptibility of male C57Bl/6 mice compared with female mice No information on psychological aspects of AN	↓body weight ↓food intake (self-starvation) ↑physical activity Loss of estrous cycle Immunodeficiency Atrophy of spleen and thymus Hypometabolism in ventral striatum and insular cortex Alteration in reward circuitry, bone physiology	Routtenberg et al. (1967) van Leeuwen et al. (1997) Hebebrand et al. (2003) Boakes et al. (2007) Casper et al. (2008) Hancock et al. (2009) Zgheib et al. (2014) Perez-Leighton et al. (2014) Achamrah et al. (2017) Welch et al. (2018)
Mild-stress ABA PROTOCOL	Sprague-Dawley rats (pups)	Males and Females	Maternal separation 1-hr feeding period 2-hr/day wheel access	↓food intake ↓body weight ↑physical activity ↓reduced anxiety in females ↑female vulnerability ↑anxiety in males	Routtenberg et al. (1967) Hancock et al. (2009) Zgheib et al. (2014) Farinetti et al. (2020)

Note: The table contains a list of observed alterations following the implement of diverse types of animal models employed in AN studies.
n.m (not mentioned).

1.3.4 Gender differences in the AN condition

The susceptibility of AN from human and animal studies has been observed to be higher in adolescent females. Heightened stress being a major risk factor for AN in the animal model (Hancock & Grant, 2009), clinical findings have also found an increased vulnerability to the behavioral and physiological effects of stress in the period when neural regions and neurotransmitter and hormone systems that modulate HPA axis activity undergo developmental change (Hancock & Grant, 2009). This period is normally at the adolescence stage (Hancock & Grant, 2009). Females become increasingly susceptible to stress during adolescence due to the contribution from various sex hormones facilitating effect on the HPA axis activity (Hancock & Grant, 2009; Iwasaki-Sekino et al., 2009). The contrary has been noted in the adolescent males where this stage is marked by the inhibitory effect of various male sex hormones on the axis, hence dampening the stress susceptibility (Hancock & Grant, 2009). There is a sex difference in the stress peptide, corticotropin-releasing factor, CRF, mRNA expression in two main stress-related regions, paraventricular nucleus of the hypothalamus (PVN) and the central nucleus of the amygdala (CeA) (Iwasaki-Sekino et al., 2009). Authors in an attempt to understand the high HPA axis activity in females and the female prevalence in AN condition, induced two main stressors, a psychological stress (PS) and an electric foot shock (FS) stress, in proestrus and estrus females and in male rats (Iwasaki-Sekino et al., 2009). They found a significantly greater PS-induced inhibition of food intake in female rats than in the male rats while electric FS-induced inhibition of food intake was almost the same in both gender (Iwasaki-Sekino et al., 2009). Acute or chronic stress is also observed to decrease CRF mRNA expression levels in males but not in females (Viau et al., 2005). The linear correlation between stress hormone levels and running wheel activity, explains in part the higher incidence of AN condition in female adolescents (Hancock & Grant, 2009; Boakes RA, 2007; Routtenberg & Kuznesof, 1967; Sakurada et al., 2000).

Despite the reasons mentioned above for the higher susceptibility in females, other animal models under the ABA paradigm have reported conflicting results (Welch et al., 2018). For instance, in the C57BL/6 male mice, there was the observation of a decreased food intake, and a higher mortality rate (Welch et al., 2018).

It is recommended that females and males be always analysed together to clarify the discrepancy amongst the different sexes in the ABA paradigm. The inclusion of both sexes could help not only to confirm the sex susceptibility but also to correctly examine the different effects in both sexes under the same protocol timeline.

1.4 STRESS IN THE EARLY LIFE ENVIRONMENT

The period surrounding birth and development of an organism has a significant impact on the shaping of physiological functions such as growth, metabolism, reproduction and immune response (Pryce & Feldon 2003; Nishi et al. 2014). Exposure during this period to stressors, including but not limited to verbal abuse or emotional neglect, does elicit transient responses necessary for survival, but to a greater extent, causes profound and long-lasting effects in the individual (Carrasco & Van de Kar, 2003; Pryce & Feldon 2003; Nishi et al. 2014). The propensity of stressors to alter structure, physiology and metabolism has led to the establishment of a strong relationship between early developmental stress and a vast number of diseases including substance abuse, schizophrenia, unipolar and bipolar depression, panic disorders and suicidal attempts (Heim & Nemeroff, 2001; Virginia Mela et al., 2012; Paternain et al., 2012).

In animal studies various modifications, both in behaviour and the neuroendocrine system in adulthood, are observed when separation-stress occurs in the early postnatal/developmental period (Bárbara Aisa et al., 2006). This period is characterized by a reduced stress response keeping glucocorticoids levels at minimal (Sapolsky & Meaney, 1986; Varga et al., 2013). A longitudinal study using histamine stress indicated a stable corticosterone levels with reduced response to stress in pups between day 3 and 16 (Sapolsky & Meaney, 1986). More importantly, it has been observed that once response to stress occurs during the early postnatal period, its suppression was highly prolonged as compared to the adult situation (Sapolsky & Meaney, 1986). Taken together, the reduced levels and response during the early postnatal period dampens the prolonged catabolic effect, hence, ensures a higher anabolic state needed for an optimal central nervous system development (Sapolsky & Meaney, 1986).

1.4.1 Maternal separation (MS)

MS, a robust animal paradigm, is used to simulate and study the emotional deficits of maternal neglect in rodents as they occur in humans (Carrera et al., 2009; Paternain et al., 2012). The HPA axis is instrumental in neuroendocrine homeostasis (Pryce & Feldon, 2003). The axis development is known to occur during the early postnatal period under optimal dam-pup interaction (Sapolsky & Meaney, 1986). The correct interaction establishes non observable events that confer salient homeostatic effects (Pryce & Feldon, 2003). Thus, the implementation of the MS procedure precipitates adverse neonatal events that predispose to the development of various kinds of diseases later in life (Nishi et al., 2014).

Length of postnatal separation day (e.g., 1 - 14 days, 2 - 6 days, 2 - 21 days, 15 - 21 days) and the duration (e.g., 5h/day, 3h/day, single 24h) of the MS procedure are different among laboratories (Biagini et al., 1998; Carrera et al., 2009; Tjong et al., 2010; Nishi et al., 2014). Based on the length, duration and mode of separation, a variety of types have been coined (Pryce & Feldon, 2003). MS usually refers to the removal of the whole litter from the dam for a couple of hours across postnatal days. Although similar to the single MS, the latter involves a single 24-hour separation. In dam and infant deprivation, the dam is removed from the home cage and pups are individually separated from the dam across postnatal days, respectively (Pryce & Feldon, 2003).

The effect of MS has a time window as reported by Carrera and Gutièrez: *'brief periods of maternal separation in rats in the first 21 days of life (15 min/day) reduced stress-response vulnerability with a decreased weight loss and activity compared to longer periods of separation (3 hours/day)'*, an analysis stemming from an altered brain metabolism (Carrera & Gutièrez, 2006).

Despite most laboratories indicating adverse effects, results obtained from different groups using the MS paradigm have been conflicting (Oomen et al., 2010; Fujimoto et al., 2014). Observations reported using this paradigm include, but not limited to, an alteration in the reactivity of the HPA axis in response to stress, an assertion that stems from the changes in DNA methylation of several gene promoters involved in the activity of the HPA axis (Jaimes-Hoy et al., 2019). Agreeably, neurobiological studies indicated an altered stress responsiveness in adult life when an early maternal neglect was present (Briere & Rickards, 2007); however, handling, which is the opposite of MS, was found to dampen the stress reactivity and is proposed as a mechanism able to reduce the susceptibility to effects induced by activity-based anorexia (Hancock & Grant, 2009). Increased pain response (with a reduced pain threshold) in response to colorectal distension stimulation was observed in two-month old maternally separated rats (Tjong et al., 2010); other effects described were a decreased capacity to cope with novelty and higher plasma level of corticosterone (Biagini et al., 1998), and a reduced reactivity of astroglia in areas known to be involved in depression and stress-related behaviour (Leventopoulos et al., 2007). Metabolically, body weight has been observed to be reduced throughout adult life when rats are given a standard chow diet following a 24-hour maternal deprivation during the early postnatal period (Mela et al., 2012). Maternally separated rats have also been observed to depict different diet preferences after weaning into the adulthood period (Paternain et al., 2012) with accumulating data suggesting a preference for a high fat over a standard chow diet (Paternain et al., 2012). Findings in the work of Caslini et al., 2015, in agreement with other longitudinal studies, confirmed the association between adverse

early environment and subsequent development of ED. The authors suggested, therefore, regarding the poor treatment outcomes, especially in AN (Carter et al., 2004), a need for early screening of abuse in such individuals (Caslini et al., 2015). In animal studies employing the activity-based anorexia model, female and male rats were observed to be hyperactive and lose bodyweight drastically, respectively, in the presence of early MS (Farinetti et al., 2020).

Effects caused by exposure to unfavorable environments take a toll on all body systems, but attenuations in the function of the cardiovascular, immune, gastrointestinal and neuroendocrine systems are often prominent (Carrasco & Van de Kar, 2003).

1.5 REWARD SYSTEM

Throughout evolution, species, including higher mammals like humans, engage in behaviours that ensure homeostatic balance and continued survival (Schultz, 1998). An essential aspect of this behaviour, aside punishment avoidance, is the pursuit of reward (Hu, 2016). Rewards encompass events geared towards consummatory and approach behaviour characterized by multiple psychological components, including liking, wanting and learning, activities essential for basic life processes (Schultz, 2010; Hu, 2016). The coherent pairing of rewards with sensory information has been found to induce high-frequency activity, a pattern that supports the long-term adjustment of synaptic transmission (Blythe et al., 2007). Several brain regions involved in the processing and anticipation of reward-related stimuli in both humans and rodents include, but are not limited to, the orbitofrontal and anterior cingulate cortex, amygdala, nucleus accumbens, and ventral tegmental area (VTA) (Berner et al., 2019).

Brain signalling functions such as aversion, salience, motivation, motor control, and in particular, food reinforcement and rewarding effects have been established to be associated with midbrain dopaminergic neuron transmission. These assertions stem from studies analyzing effects of lesions, receptor blocking, electrical self-stimulation, and drugs of abuse (Schultz, 1998; Tritsch et al., 2014; Lammel et al., 2015; Berner et al., 2019).

The midbrain DA system comprises a complex system of DA neuron subtypes, principally in the substantia nigra (SN) and VTA, together with almost 30% GABA and 2-3% glutamate neurons (Lammel et al., 2015; Ferrario et al., 2016).

These neurons have glutamatergic NMDA and AMPA receptors, which modulate dopamine burst response to reward-related stimuli (Schultz, 2010). Two main pathways characterize the transmission of the midbrain DA neurons: nigrostriatal and mesocorticolimbic pathways. The former sends projections from the substantia nigra pars compacta (SNpc) mainly to the caudate and putamen (dorsal striatum), allowing motor behaviour modulation. In contrast, the latter sends projections from the ventral tegmental area (VTA) to the ventral striatum and regions of the prefrontal cortex to modulate cognition, motivation and reward (Kapur & Remington, 1996; Tritsch et al., 2014).

1.5.1 Dopaminergic system in AN

In the presence of food shortages, animals physiologically respond by adopting a foraging strategy intended to create a positive energy state (Sodersten et al., 2016). This response is observed to impact stress neuropeptides, resulting in increased stress response (Holly et al., 2015). The location of the corticotropin-releasing factor (CRF) and its receptors, precisely the CRF-R2, within the VTA consequently activates the dopaminergic system (Sodersten et al., 2016; Holly et al., 2015) and its termination sites such as the nucleus accumbens (Sodersten et al., 2016). Repeated stress exposure arising from caloric restriction and intense physical activity, as in anorexics, is not surprising in sustaining altered adaptations in the dopaminergic system where dieting and physical activity become rewarding at the expense of food intake (Sodersten et al., 2016; Holly et al., 2015). The prevailing characteristics of AN indicate a remarkable anomaly in the rewarding value of food and exercise, parameters known to be under the control of the dopaminergic pathway (DSM-5).

Most neuroimaging studies have confirmed changes in activation patterns within the brain reward circuit in AN patients. A study that used a positron emission tomography (PET) imaging with the radioligand [^{11}C]raclopride to compare D2/D3 receptor function between controls and women recovered from AN observed an increased D2/D3 receptor binding in recovered AN subjects (Frank et al., 2005). The authors explained the increased receptor activity in the recovered subjects as a 'persistent scar from the illness' due to changes in dopamine activity at critical developmental periods (Frank et al., 2005). Similarly, the major metabolite of dopamine in humans, homovanillic acid, was reduced in cerebrospinal fluid of restricting-type AN patients and persisted even after more than a year of recovery compared with healthy control women (Kaye et al., 1999). The possible altered metabolism of DA, as suggested by the authors, contributes to the maintenance of the disorder and high relapse after recovery (Kaye et al., 1999). Anhedonia, characterized in part by the aversiveness to food-related stimuli, in AN patients has also been observed to be regulated by the mesolimbic dopamine system (Foldi et al., 2017). Analysis of responsivity to images of thin bodies in a reward-related region, the ventral striatum, was shown to be higher in AN compared to control individuals (Frank et al., 2012) confirming the little to no pleasure in anything in life aside the pursuit of body weight loss in AN patients (Kaye et al., 1999).

Dopamine analysis in animal models has been examined in several research works employing antipsychotics. Mistlberger and Mumby, 1992, using ablation and pharmacological procedure, reported a decline in activity levels after administering a higher-dose dopamine D2 receptor

antagonist (Mistlberger & Mumby, 1992). Agreeably, at lower concentrations, the non-selective antagonist for the D1/D2 receptor, cis-flupenthixol, resulted in activity levels in female Wistar ABA rats (Verhagen et al., 2009). The D1 dopamine receptor is implicated in cognition, rewarding and motor activating effects and is altered in diseases with profound cognitive defects. Performing an intracerebroventricular injection of the D1 and D2/3 DA receptor agonists, SKF-82958 and quinpirole, respectively, authors observed a further increase in activity levels of food-restricted Sprague Dawley male rats compared to ad lib fed group (Carr et al., 2003).

As noted, dopamine neurotransmission is important in reward seeking behaviours both in human and animal studies. Although it works in tandem with multiple systems, its contribution to hyperactivity in the AN condition cannot be overlooked due to reduction of motor behaviour upon selective dopamine lesions and antagonists administration (Kelly et al., 2005). However, how the activation of the dopaminergic mesolimbic rewards pathway reinforces physical activity during food restriction and, in the contrary, in anorexic patients such as in ABA rodents is still in part elusive.

1.5.2 Serotonergic system in AN

The serotoninergic system, with its neurons widely distributed in strategic anatomical locations, including the gut, seems to be implicated in the pathogenesis of the AN disorder (Blundell, 1984; Lesch & Merschdorf, 2000; Mann JJ, 1984). These neurons have their cell bodies densely located in the raphe nuclei and project towards the forebrain, striatal and limbic regions (Kaye et al., 2005), playing essential roles in satiety and mood regulation (Haleem, 2017). Although not directly related to the reward system, projection of serotonin neurons from the raphe nucleus to the SN and ventral tegmentum allows for the modulation of dopamine-mediated behaviours (Kapur & Remington, 1996; Carta, Fadda & Stancampiano, 2006; Haleem, 2017). In fact, psychostimulants administered to decrease or increase serotonin tone has been observed to increase or decrease, respectively, the locomotor activity in rats (Carta, Fadda & Stancampiano, 2006).

Serotonin firing has been reported to be inversely related to dopamine release (Kapur & Remington, 1996), thus increasing the availability of 5HT should reduce the hyperactive effect of dopamine in AN. However, in AN individuals the amount of the primary amino acid required for serotonin synthesis, i.e. tryptophan (TRP), is below normal levels due to food restriction, especially carbohydrate-rich diet. Agreeably, authors administering a TRP – deficient diet in male Sprague Dawley rats observed a reduction of both striatal content of 5HT and its metabolite, 5-

hydroxyindolacetic acid (5-HIAA) (Carta, Fadda & Stancampiano, 2006). Also, hyperactivity induced by amphetamine administration was significantly higher in the deficient rats than the controls and subjects with a TRP-replenished diet (Carta, Fadda & Stancampiano, 2006).

The cell bodies of 5-HT neurons are located in the raphe nuclei of the midline brainstem and project to cortex, striatal, and limbic regions. AN is often associated with anxiety-related disorders and co-morbid with depression (Hancock & Grant, 2009), allowing psychotropic medications such as serotonin-selective reuptake inhibitors, SSRIs. These drugs have recorded excellent results in reducing depressive symptoms, although no effect on the food-intake restriction behaviour has been observed (Madra & Zeltser, 2016). SSRIs are potent desensitisation agents, especially on the 5-HT1A autoreceptor (Haleem, 2017); they work better when sufficient TRP is available, which is essential for 5-HT production and its metabolites. In AN individuals, the concentration of TRP is below normal levels hence 5HT and its metabolites. In addition, the activity of the 5-HT1A autoreceptor is very high (Haleem, 2017). The reduced TRP availability and enhanced activity of the autoreceptor do not allow the SSRI's to have their therapeutic effect. In recovered individuals where the concentration of extracellular 5HT is sufficient due to normalised feeding and weight restoration, it allows the inhibitory action on the autoreceptors to be enhanced, allowing the release of a higher concentration of 5HT, hence, its consequential effect (Haleem, 2017).

AN, and other psychiatric disorders, is characterised by a loss of serotonin function linked to a reduction in 5-HT2A receptor binding in the frontal, parietal and occipital cortices both in the disease state and after weight recovery when compared to healthy controls (Foldi et al., 2020). Decreased concentration of the primary 5-HT metabolite (5-HIAA) is observed in the cerebrospinal fluids (CSF) of AN patients (Foldi et al., 2020).

CHAPTER 2: AIM OF THESIS

Anorexia nervosa (AN) is a psychiatric disorder denoted by extreme caloric restriction, hyperactivity (in most patients) and a global endocrine dysregulation predominantly in women and adolescent girls. A principal animal model that combines physical activity and reduced food intake employed in AN's study is activity-based anorexia (ABA). The model's efficacy in recapitulating the core features of AN in humans has paved the way for studying the parameters involved in the development and maintenance of the disorder. Although factors involved in the evolution of AN is diverse, stress resulting from the adverse early environment has mainly been analysed. In animal models, this condition is recapitulated using the maternal separation paradigm, which involves the isolation of the whole litter from the dam for some time across several postnatal days.

Although there is substantial literature on the effect of adverse early environment on the susceptibility of AN in human and animal studies (Carrera et al., 2006), not much work has been focused on analysing the impact of the different activity periods in the ABA protocol in the presence of early maternal separation. For instance, the food anticipatory activity and postprandial activity periods have been observed to contribute to the vulnerability to the ABA protocol (Wu et al., 2014; Klenotich et al., 2012). Hence, combining these two essential protocols, we aimed to analyse which activity period(s) confer a higher susceptibility to the pathological weight loss observed in our ABA protocol.

Secondly, changes in subjects' behaviour following induction of ABA protocol in the presence or absence of the maternal separation stressor were analysed. Analysis of exploratory and anxiety-related behaviours commonly seen in AN individuals was assessed using the elevated plus maze and open field tests.

Thirdly, specific brain regions known to sustain the pathophysiology of AN condition both in humans and animals including the dopaminergic and serotonergic systems were analysed.

In addition, since AN disorder is present in both genders (with a higher female prevalence), we employed male and females to throw more light on the sex differences in the development of the AN disorder. The inclusion of both sexes in this thesis would contribute to bridging the gap between the correlations of AN effects in different sexes.

CHAPTER 3: EFFECT OF DAM-PUP SEPARATION ON MATERNAL BEHAVIOUR AND EARLY DEVELOPMENTAL PARAMETERS

3.1 INTRODUCTION AND AIM OF EXPERIMENT

As mentioned in the general introduction (chapter 1), the emotional deficits of maternal neglect in rodents as they occur in humans can be evaluated using the MS procedure (Carrera et al., 2009; Paternain et al., 2012). Among the many alterations that can arise from an adverse early environment there is the inability to respond correctly to stress during adult life, a condition able to increase the incidence of developing psychiatric disorders (Pryce & Feldon 2003).

In animals, 3 hours per day separation in male pups was observed to result in a higher anxiogenic effect upon exposure to stress in adult life (Kalinichev et al., 2002). Changes in response to the effect of the ABA protocol when a maternal separation is present have also been observed. Among the many effects documented are the modulation in anxiety-like behaviours when female maternally separated were compared to non-maternally separated rats. Also, drastic body weight loss and enhanced activity levels were observed in ABA male and female rats, respectively, in the presence of an early maternal separation (Farinetti et al., 2020).

An optimal dam-pup interaction, especially in the early postnatal days is crucial to the survival and development of the pups (Ferreira et al., 2012; Sapolsky & Meaney, 1986). When pups are separated from the dam to a new environment, they become exposed to new odours and different environmental temperatures. The call and response time of ultrasonic vocalisations becomes altered (Rees & Fleming, 2001). Although brief periods of separation have been observed to confer less fear-like behaviour, not the same can be said of long maternal separation (Kalinichev et al., 2002).

This part of the experiment aimed to observe the interactions between the dam and the pups during the MS procedure. We also sought to analyse the effect of these changes, if any occurred, on the evolution of the bodyweight of the rats as they develop, especially on the weaning day. As a final part, we analysed the food intake and effect of the separation protocol on wheel activity before the induction of the ABA protocol.

3.2 MATERIALS AND METHODS

We obtained 6 males 12 primiparous females outbred Sprague Dawley (SD) rats from the Charles River Laboratories (Lyon, France). The rats were housed in separate cages according to sex for a one-week quarantine period. Male and female rats were paired in a ratio of 1:2 for ten days, and afterwards, separated into individual Plexiglas boxes (21 x 45 x 24 cm) with a metal mesh lid used to place food and water bottles. The floor of the cages was lined with only wood shavings without any other enrichment. Females remained separated until parturition.

The animals were kept in a temperature-controlled room at 21 ± 2 ° C, 60% of relative humidity, and a 12-hour light-dark cycle, beginning the light period at 8:00 am. All applicable international and national guidelines for the care and use of animals were followed (European Union Council Directive 2010/63; Spanish Royal Decree 53/2013), and the procedures performed were under institutional ethical standards. A schematic representation of the experimental design is depicted in figure 1.

3.2.1 Maternal separation

On the day of birth, designated as postnatal day (PND) 0, we chose litters for maternal separation and non-handled. The manipulation was conducted once daily, in the morning, from PND 1 to PND 15 (Hancock & Grant, 2009; Farinetti et al., 2020). Briefly, we removed MS pups (a total of 25 and 22 animals for females and males, respectively, divided into CON (ad lib feeding without wheel access), DIET (food restriction without activity wheel) and ABA (activity wheel with food restriction) groups; figure 1) from their birth cage, and each litter was placed separately in a smaller cage with bedding material inside an incubator for 3 hours. Prior to removing MS pups, the dams whose pups had to be separated were removed from their home cages and put into single cages (the same cage every day for each dam). The pups were then removed from the home cage and put together in a small cage (the same cage every day). Pups were collected with some amount of litter beddings to maintain the scent in the home cage. The cage with the pups was placed inside a 48 x 30 x 30 cm glass incubator (OVAN, Lovango s.l. Barcelona, Spain) for 180 minutes (3 hours). The incubator had a humidifier system to control the humidity of the chamber and an in-built thermostat that allowed the regulation of temperature to suit the ages of the pups. After the 3 hours, pups were immediately put back into the home cage, and the dam returned to

them. We observed for some minutes if the dam accepted its pups by looking out for behaviours such as picking the pups with its mouth to a particular spot, licking or covering them with its body.

From PND 1 to 15, pups were placed in the incubator maintained at 55-58% humidity. The temperature was kept between 33-34 °C from PND 1 to 7 and 31-32 °C from PND 8 to 15. This modulation in temperature was necessary to ensure pups' survival (Jans & Woodside, 1990). During the 15 days of separation, non-handled group (a total of 25 and 19 animals for females and males, respectively) were left undisturbed with their mothers until weaning.

On postnatal day (PND) 21, the non-separated and maternally separated groups (MS is used in the name of the group as a suffix to distinguish it from non-separated groups) were weaned and sexed. We took the body weight and subdivided the subjects into same-sex cages, 3-4 rats per cage, totalling about 15 cages. Male (abbreviated as M) and female (abbreviated as F) rats were assigned to sex-specific groups: In this way, females were divided into FCON (N = 8), FDIET (8), FABA (N = 7), MSFCON (N = 9), MSFDIET (8), MSFABA (N = 8), and males into MCON (N = 5), MDIET (7), MABA (N = 7), MSMCON (N = 7), MSMDIET (7) and MSMABA (N = 8), see figure 1. We divided animals into respective groups based on equal or similar body weights in order to avoid differences in performance in the ABA protocol due to differences in body weights. Food and water were available ad libitum until animals underwent the experimental procedure. Litter size ranged from 14 to 16 pups per dam. In order to prevent any additional stress from standardization, curling of litters was not performed. Each litter remained with its dam until the weaning period. We used the standard weaning stage in rodents because the early or late weaning period contribute to variability in behavioural and neurophysiological tests (Bailoo et al., 2020).

From PND 36 to 40, we performed the habituation process. Animals of all groups were weighed and given weighed amount of food (800 g for males per cage and 700 g for females per cage) on P36, 38 AND 40. Food ingested was measured within two-day intervals (i.e. P38 and P40) and body weights recorded. This procedure was essential to control their growth and estimate a baseline body weight and understand how much they ate in a normal condition. The total amount of food ingested was divided by the number of rats per cage to estimate the quantity consumed.

On PND 41-42, rats to be assigned to the ABA group were removed from their home cages and were put for two (2) hours (Carerra et al., 2009) in individual cages with a running wheel to fit the new environmental context. This pre-exposure was necessary to prevent the neophobic effect which has been observed to affect activity on the running wheel during the ABA protocol (Carrera et al., 2006).

3.2.2 Statistical analysis

Data collected during this section of the experiment were analysed mainly by repeated-measures ANOVA. All analyses were performed after verifying the normality of the data with the Shapiro test and were followed, if the main effects were significant, by the Student-Newman-Keuls (SNK) post hoc test. The SPSS 24.0 program was used to calculate the *p* values, and the significance threshold was set at $p < .05$.

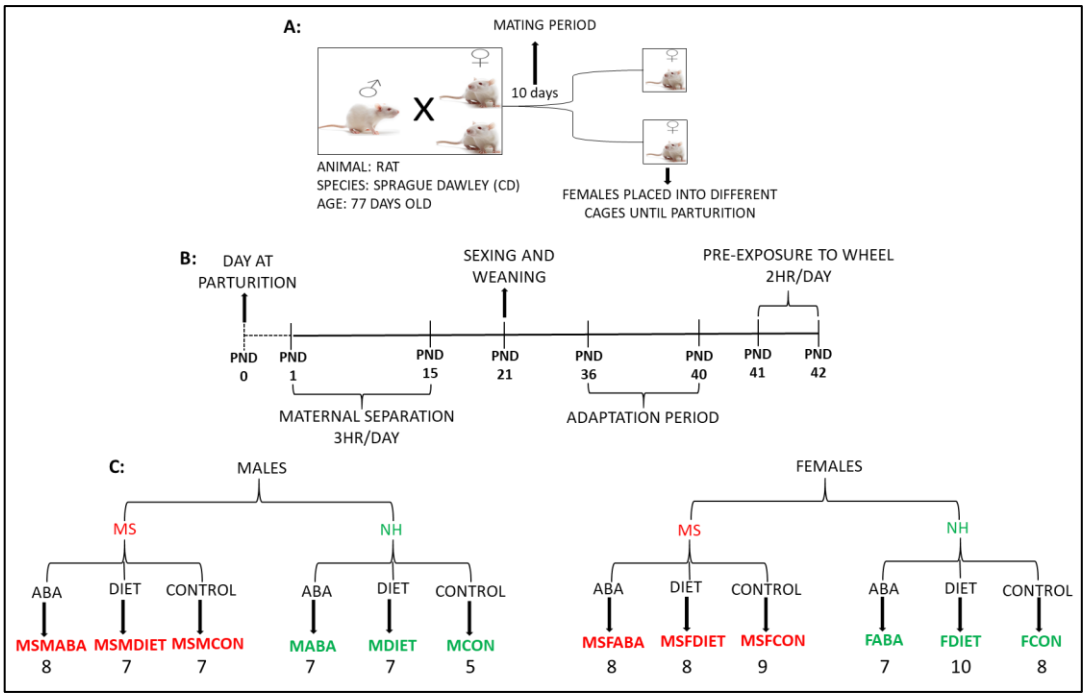


Figure 1. Summary of experimental protocol and groups analysed prior to the ABA protocol.
The figure shows the type of pairing used during mating (**A**), protocol timeline (**B**) and groups analysed (**C**).
PND (postnatal day), MS (maternal separation), NH (non-handled), ABA (activity wheel with food restriction), DIET (food restriction without activity wheels), CONTROL (ad lib. feeding and without wheel access).

3.3 RESULTS: PRIOR TO ABA INDUCTION

3.3.1 Maternal behaviour towards pups upon reunion

The video recording (15-20 minutes) taken upon the reunion of pups with the dam, by observation, revealed that:

As recorded in Table 2, from PND 1 to PND 5, dams expressed high levels of non-pup directed behaviours such as cage exploration and self-grooming. Pup directed behaviours such as retrieval, corporal and anogenital licking and nest creation were minimal. No signs of aggression were observed during these days.

From PND 6 to PND 15, dams expressed a higher level of pup directed behaviours, especially crouching and anogenital licking. Non-pup directed behaviour was minimal.

More importantly, we observed unequal maternal attention towards the pups across the entire separation days.

Table 2: Observed maternal behaviour towards pups upon reunion during separation days

BEHAVIOUR/DAYS	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
NON-PUP DIRECTED															
CAGE EXPLORATION	3	3	3	3	3	1	1	1	1	1	1	1	1	1	1
SELF-GROOMING	3	0	3	3	3	0	0	1	0	0	1	0	0	0	0
SCRATCHING	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0
PUP DIRECTED															
NEST BUILDING	NO	YES	YES	YES	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO
LATENCY_RETRIEVAL	3	1	1	1	1	1	1	1	1	0	0	0	0	0	0
DURATION_RETRIEVAL	1	1	1	1	1	1	1	1	1	0	0	0	0	0	0
LATENCY_CROUCHING	3	3	3	0	3	1	2	2	1	1	1	1	1	1	1
DURATION_CROUCHING	1	1	2	0	1	3	3	3	3	3	3	3	3	3	3
ANOGENITAL LICKING	0	0	2	1	0	3	3	3	3	3	3	3	3	3	3
OTHER BEHAVIOUR(S)															
AGGRESSION	NO	NO	YES	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO	NO

Note: Average frequency of maternal behaviour of all dams towards pups. Recording was made between 15-20 minutes. The following behavioural responses were recorded (Ferreira et al., 2012; Yamamura & Sensui, 1999):

- Self-grooming: The frequency of self-grooming to the nasal region by fore legs.
- Scratching: The frequency of body-scratching with a hind leg.
- Nest building: Involves the dam carrying nest material with her mouth, or pushing it with her paws or snout, to the (potential) nest site
- Latency to retrieval: The latency to retrieval of the first pup after the return of the litter to the cage.
- Duration of retrieval: The duration for carrying all or more than 6 pups into the nest after the onset of retrieval.
- Crouching: The time the mother spent crouching over the pups to facilitate suckling, remaining immobile except for occasional postural adjustments.
- Licking: The duration of licking in the anogenital region of the pups.

0 (Absent behaviour), 1 (Lower frequency), 2 (Moderate frequency), 3 (Higher frequency)

3.3.2 Body weight on the weaning day

As illustrated in Table 4, a two-way ANOVA revealed no main effect either by sex ($F_{(1,87)}$ = 0.039, p > 0.05) or stress ($F_{(1,87)}$ = 1.783, p > 0.05) when subjects were weighed at PND 21. However, there was a trend of increased bodyweight for both male and females non-maternally separated compared to respective maternally separated groups.

3.3.3 Food intake during adaptation period

Although there was no main effect given by the neonatal treatment when food was freely available, the statistical analysis revealed a main effect of sex on both days analysed (PND 38: $F_{(1,87)}$ = 70.867, p < 0.001, PND 40: $F_{(1,87)}$ = 34.382, p < 0.001, Table 3). Following the Student Newman-Keuls (SNK) test, a higher amount of food ingested was observed for NHMALE and MSMALE compared to respective female groups (PND 38: $F_{(3,90)}$ = 23.669, p < 0.001, PND 40: $F_{(3,90)}$ = 11.792, p < 0.001, figure 2).

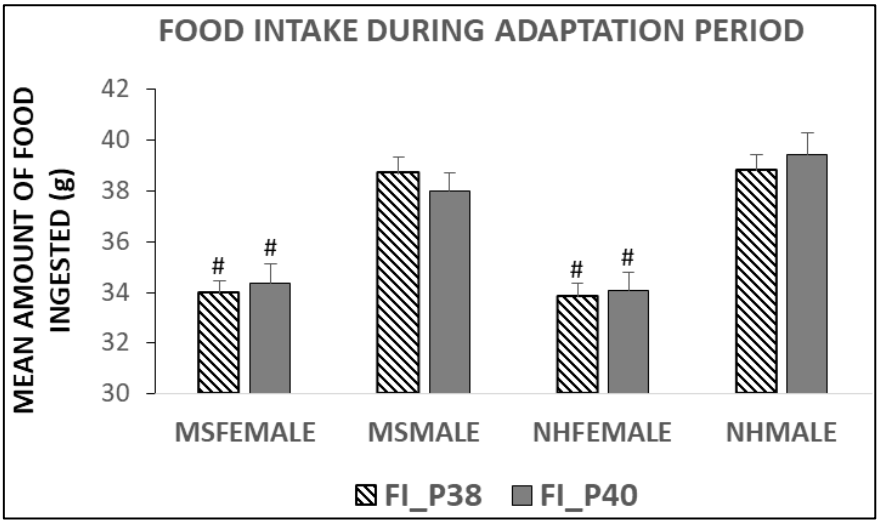


Figure 2: The graph represents data on the food ingested (expressed in grams) on selected days during the adaptation period. The means and the standard error of the mean (SEM) of chow consumed are represented in the graphs.
Number sign (#) = (p < .05) sex differences in the same groups (NHFEMALE vs. NHMALE)
NH (non-handled), MS (maternal separation).

Table 3: Two-way univariate ANOVA main effects

PARAMETERS	SEX (M/F)	STRESS (MS/NH)	INTERACTION
FEEDING TEST AT PND 38	$F_{(1,87)}$ = 70.867, p < 0.001	n.s	n.s
FEEDING TEST AT PND 40	$F_{(1,87)}$ = 34.382, p < 0.001	n.s	n.s

Note: The values of F and p were obtained through a two-way repeated-measures ANOVA, used to analyse the possible effects of sex (M vs F), stress (MS vs non-handled) and their interactions on parameters collected prior to the period of the ABA protocol.
F (females), M (males), MS (maternal separation), NH (non-handled), n.s (not significant).

3.3.4 Weight evolution prior to ABA induction

The difference in body weight was not observed until PND 40. Although there was a main effect of sex on PND 38 ($F_{(1,87)}$ = 6.097, p < 0.05, Table 4), the post hoc analysis did not reveal any significant difference, p > 0.05. On PND 40, a two-way ANOVA revealed a main effect of sex ($F_{(1,87)}$ = 6.068, p < 0.05) and stress ($F_{(1,87)}$ = 5.164, p < 0.05, Table 4). The SNK post hoc test showed a higher weight for NHMALE compared to MSMALE ($F_{(3,90)}$ = 4.003, p = 0.010, figure 3). Also the NHMALE weighed heavier compared to MSFEMALE and NHFEMALE groups ($F_{(3,90)}$ = 4.003, p = 0.010, figure 3).

On PND 43, before the ABA procedure commenced, we observed a main of sex ($F_{(1,87)}$ = 13.033, p < 0.001) with NHMALE weighing heavier than both NHFEMALE and MSFEMALE ($F_{(3,90)}$ = 5..141, p = 0.003, figure 3).

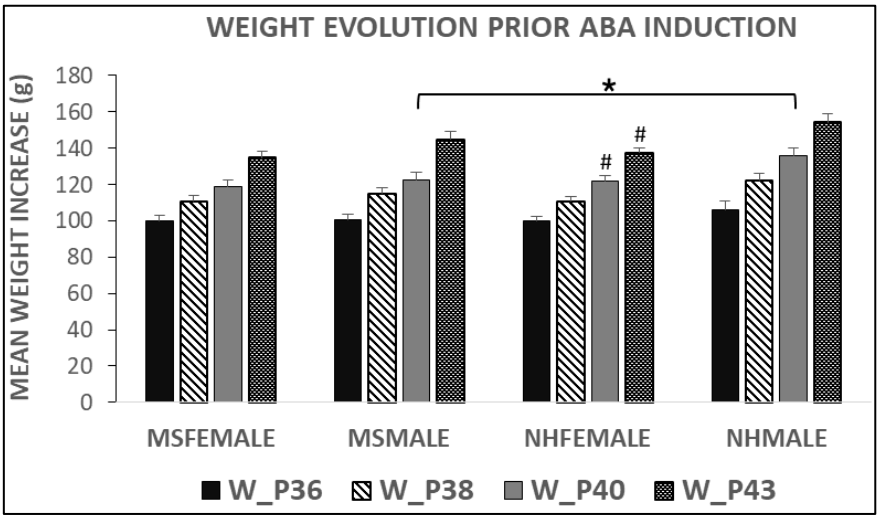


Figure 3: The graph represents data on subjects bodyweight (expressed in grams) on selected days during the adaptation period. The means and the standard error of the mean (SEM) are represented in the graphs.
Asterisk (*) = (p < .05) among the differently treated groups (MSMALE vs NHMALE)
Number sign (#) = (p < .05) sex differences in the same groups (NHFEMALE vs NHMALE)
NH (non-handled), MS (maternal separation).

Table 4: Two-way univariate ANOVA main effects

PARAMETERS	SEX (M/F)	STRESS (MS/NH)	INTERACTION
BODYWEIGHT AT PND 21	$F_{(1,87)}$ = 0.039, p > 0.05	$F_{(1,87)}$ = 1.783, p > 0.05	n.s
BODYWEIGHT AT PND 36	n.s	n.s	n.s
BODYWEIGHT AT PND 38	$F_{(1,87)}$ = 6.097, p < 0.05	n.s	n.s
BODYWEIGHT AT PND 40	$F_{(1,87)}$ = 6.068, p < 0.05	$F_{(1,87)}$ = 5.164, p < 0.05	n.s
BODYWEIGHT AT PND 43	$F_{(1,87)}$ = 13.033, p < 0.001	n.s	n.s

Note: The values of *F* and *p* were obtained through a two-way ANOVA, used to analyse the possible effects of sex (M vs F), stress (MS vs non-handled) and their interactions on parameters collected prior to the period of the ABA protocol.
F (females), M (males), MS (maternal separation), NH (non-handled), n.s (not significant).

3.3.5 Activity wheel analysis prior to ABA protocol

A two-way ANOVA revealed a main effect of sex ($F_{(1,26)}$ = 11.072, p < 0.05, Table 5) but not of stress or the interaction between them. Activity levels were higher in females with respect to males, with a significant effect in the female group without neonatal separation. In addition, among females, the neonatal treatment significantly reduced activity levels on the first day as represented in figure 4.

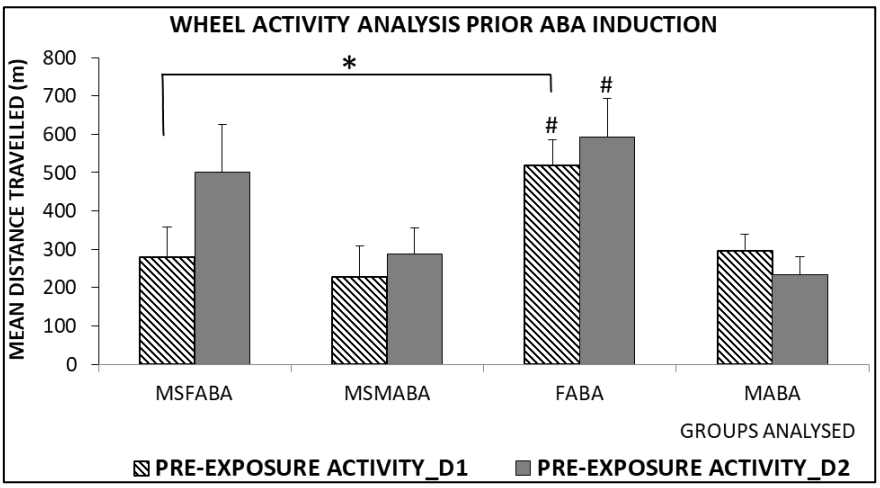


Figure 4: The graph represents data on the distance travelled on running wheel (expressed in meters) during the pre-exposure period. The means and the standard error of the mean (SEM) are represented in the graphs.
Asterisks (*) indicate significant differences (p < .05) among the differently treated groups (ABA vs. MSABA), and number signs (#) indicate sex differences in the same groups (p < .05, FABA vs. MABA, MSFABA vs. MSMABA).
MS (maternal separation), F (females), M (males), ABA (activity-based anorexia).

TABLE 5: Two-way repeated-measures ANOVA main effects on wheel analysis prior to the ABA protocol.

PARAMETER	SEX (M/F)	STRESS (MS vs NH)	SEX * STRESS
PRE-EXPOSURE WHEEL ACTIVITY (m)	$F_{(1,26)}$ = 11.072, p < 0.05	n.s	n.s

Note: The values of *F* and *p* were obtained through a two-way repeated-measures ANOVA, used to analyse the possible effects of sex (M vs. F) and stress (MS vs. non-handled) and their interactions on parameters collected during the pre-exposure days.
F (females), M (males), MS (maternal separation), NH (non-handled), ABA (activity-based anorexia), n.s (not significant).

3.4 DISCUSSION

In the first part of my study, the impact of the MS protocol was considered. Recordings during the separation protocol indicated an inconsistency in the dam-pup interaction across postnatal days. Although not significant, a trend of increased body weight was observed in both non-handle male and female groups compared to respective MS groups. During the adaptation period, food intake and weight analysis appeared consistent with the literature, that is, higher in males to females (Hancock & Grant, 2009). Finally, pre-exposure to activity wheels revealed higher levels in females, with the separation protocol seeming to provide a modulatory effect in most groups.

Maternal behaviour in rodents consists of multiple connected elements meant to establish an optimal environment necessary for neurodevelopment and behavioural responses during the most vulnerable stage of pups' life (Pryce et al., 2003; Paternain et al., 2012). These behaviours are established generally during the late stage of gestation and have been shown to be under hormonal control (Yamamuro & Sensui, 1999). Blockade of endogenous hormones has been observed to hamper lactation (Yamamuro & Sensui, 1999). More importantly, the continued expression of these behaviours is bi-directional, dependent not only on the dam but also on littermates (Pryce et al., 2003). For instance, when the dam assumes the arched back posture during nursing, the milk is let out when the littermates make enough suckling stimulation (Pryce et al., 2003). The increased incidence of non-pup directed behaviours observed during the first week of separation could increase stress levels in both littermates and the dam due to the disrupted interaction. Littermates separated from dam typically emit higher ultrasonic vocalisations as a sign of distress (Varga et al., 2015). This vocalisation is reduced upon anxiolytic drugs' administration. In my analysis, the amount and the importance of the observed dam passive behaviour are unclear; other works have also indicated that a minimal amount of stress in the early postnatal period is necessary for normal development (Pryce et al., 2003).

Bodyweight analysis at the weaning period is instrumental in allowing the investigation, if any, of the effect of MS on the bodyweight of rats. Conflicting results have been obtained, with some indicating differences (Carrera et al., 2009) and others recording no differences in body weight (Farinetti et al., 2020). As indicated in this work, we observed a trend of increase for the non-handled group but no significant differences between the two groups, suggesting that maternal separation may not necessarily confer negative consequences but may contribute to animal growth. Although minimal manipulation is necessary during the postnatal period for suitable development in mammals (Cadji et al., 1998; Carrera et al., 2009), the discrepancies usually

observed across different laboratories could be explained by the differences in the type of separation protocol employed.

Results concerning susceptibility to the ABA protocol have been varied, especially concerning activity analysis and percentage body weight loss. According to the literature, subjects directly introduced to the ABA protocol without an adaptation period have altered fear response due to the novel environment (Carrera et al., 2006). To avoid this, we conducted activity wheel pre-exposure analysis to understand how much rats will run when not food-restricted and, more importantly, investigate the effect of the separation protocol. As reported, activity levels were higher in females than males, and the separation protocol in females modulated activity levels, especially on the first day analysed.

In a nut shell, this part of the experiment has demonstrated that maternal behavioural pattern do change when littermates are separated for long periods especially in the initial days of the separation, As to what extent these changes affect the littermates is still being debated since some level of stress is necessary for normal development (Sapolsky & Meaney, 1986). Nevertheless, the separation protocol appeared to not reduce body weight evolution and food intake but, more importantly, seemed to provide a modulatory effect on activity on the running wheel.

CHAPTER 4: INFLUENCE OF EARLY MATERNAL SEPARATION ON THE SUSCEPTIBILITY TO THE ACTIVITY-BASED ANOREXIA MODEL

4.1 INTRODUCTION AND AIM OF EXPERIMENT

One of the most effective ways of studying any human disease is the ability to simulate it in an animal model (van Leeuwen et al., 1997). The ABA protocol is a robust paradigm widely employed in analysing the core features of AN, aside from the psychological components of the disorder (Hancock & Grant, 2009; Chowdhury et al., 2015, Farinetti et al., 2020). The components of the protocol, which include food restriction and access to an activity wheel, allow for the analysis of the contribution of these parameters in the evolution and the maintenance of anorexia in animals (Chowdhury et al., 2015).

This part of the experiment aimed to analyse the effect of food-restriction alone or food-restriction and wheel access on body weight loss in the presence or absence of maternal separation. Most previous works focused on the effect of an adverse early environment or from the ABA protocol has either been done separately, employed in only one sex or performed at different stages for males and females (Carrera et al., 2006 & 2009, Wu et al., 2014). Due to the multifaceted nature of the AN disorder in humans, which at many times occur in unison as indicated in Chapter 1, paragraph 1.2, implementing the ABA protocol in the presence of a maternal separation does allow to recapitulate the disorder in an efficient way hence, the ability to understand the contribution of each component.

A salient aspect of this experiment is implementing the MS and ABA protocol in both male and female subjects. Although a higher female prevalence is observed in AN, comparing both sexes simultaneously as undertaken in our work will help to decipher susceptibility differences in both sexes. This knowledge suggestively can help tailor treatment or therapies meant to ameliorate the disorder.

Last but not least, animals engaged in higher activity levels have a dampened reinforcement value of food: activity and feeding become rewarding and aversive, respectively (Ratnovsky & Neuman, 2011). The consequential effect of high activity levels on caloric depletion, suggestively, sustains the pathological weight loss observed in both human and animal studies (Wu et al., 2014). In the ABA protocol, not all periods confer the same activity levels; in fact, the higher the activity levels during certain periods the higher the vulnerability to the protocol (Wu et al., 2014). Activity, generally speaking, has its positive impacts, such as enhancing the wellbeing of an individual; hence, eliminating activity is not an ideal way of helping AN individuals but reducing intensity in specific periods known to underline the drastic weight loss. Therefore, we analysed activity during different periods to understand which period(s) sustains the hyperactivity in our subjects.

4.2 MATERIALS AND METHODS

4.2.1 Animals

See Chapter 3, paragraph 3.2.1, figure 1

4.2.2 ABA protocol

At the end of the habituation process, rats of both the maternally separated and non-handled groups were divided into ABA, DIET, and CONTROL (figure 6). The ABA, i.e. the experimental group with activity, had 22 hours of access to the activity wheel and 1-hour access to food. The DIET group had the same exposure time to food (i.e. 1 hour) but without access to the activity wheel. Finally, in the CONTROL group, rats had access to food ad libitum without any running wheels. We provided all groups with water ad libitum during the entire experimental period.

At PND 43, we began the ABA protocol. We considered this day as DAY 0 of the protocol. We established the baseline weight of each rat. The ABA and DIET group were food-restricted at 11:00 am, and the ABA group were allowed access to the wheels at the same time until the next day at 9:00 am. We measured daily the food ingested, body weight, number of wheel rotations, and time spent on the running wheel during the ABA protocol. The running wheel comprised a Plexiglass box (21 x 45 x 24 cm), with a hole in the left wall where an activity wheel (9 cm wide and 34 cm diameter) was attached (figure 5). We granted access to the wheel by sliding manually the flap used to open and close the hole. Each wheel had a magnet attached to its side that closed an electric circuit and counted a turn when passed by the sensor. The wheel had a brake mechanism to prevent movement during the food and break time (figure 5). We used a MEDPC program for Windows to control the brake mechanism and the number of completed laps. The ABA group was removed from the protocol after attaining the criterion body weight loss, 20 – 25 %, of baseline body weight.

4.2.3 Statistical analysis

Data collected during the experimental procedure included body weights, daily food intake, and wheel revolutions. We analysed these data by two-way repeated-measures ANOVA with sex and treatment as independent variables. All analyses were performed after verifying the normality of the data with the Shapiro test and were followed, if the main effects were significant, by the Student-Newman-Keuls (SNK) post hoc test. For each single-sex group, a 2x2 factorial design was used for the test procedure: One factor was whether separated postnatally (MS versus NH), and the other was whether the rat had access to a running wheel (ABA versus DIET). The SPSS 24.0 program was used to calculate the *p* values, and the significance threshold was set at $p < .05$.

Figure 5: Running wheel apparatus used for ABA analysis.

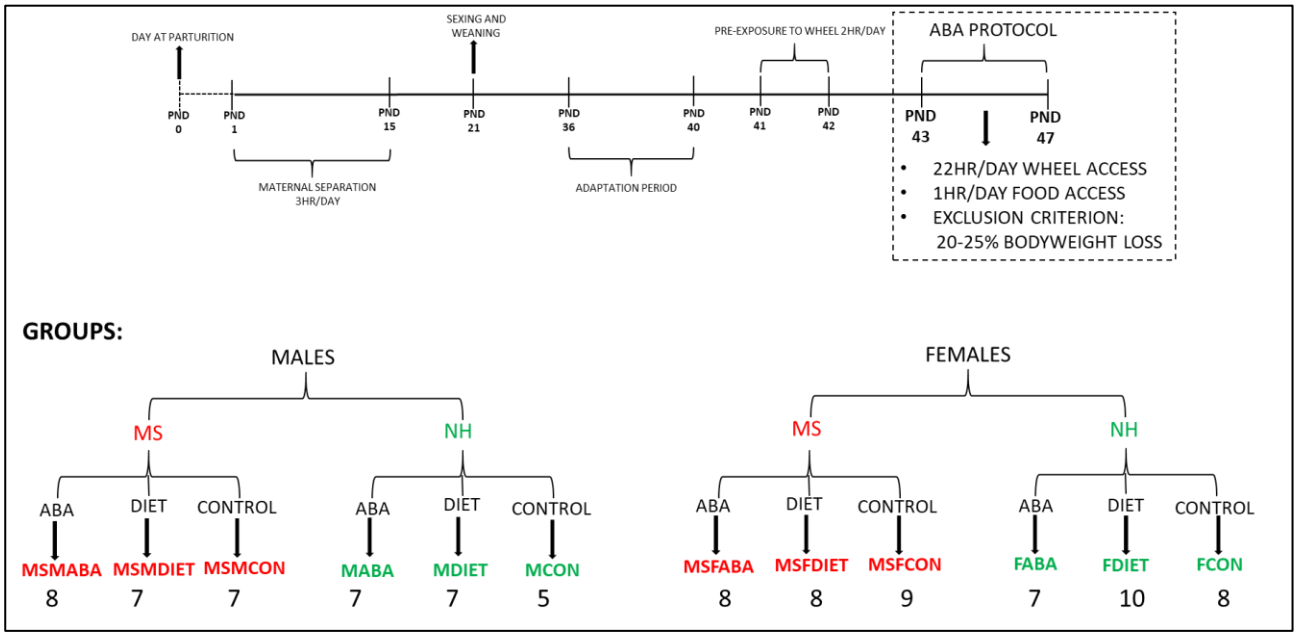


Figure 6: Summary of experimental protocol and groups analysed during the ABA protocol.
Daily measurements of food ingested, bodyweight, time spent on wheel and wheel activity were taken daily during ABA protocol.
PND (postnatal day), MS (maternal separation), NH (non-handled), ABA (activity wheel with food restriction), DIET (food restriction without activity wheels), CONTROL (ad lib. feeding and without wheel access).

4.3 RESULTS: DURING ABA INDUCTION

4.3.1 Food ingested

A main effect on sex ($F_{(1,78)}$ = 18.805, p < 0.001), treatment ($F_{(5,79)}$ = 197.227, p < 0.001) and their interaction ($F_{(5,79)}$ = 7.380, p < 0.001) was observed when all groups were combined, Table 6 (A). The Student Newman Keuls post hoc test showed a significantly higher amount of food intake in male compared to female control groups, p < 0.001, on all days analysed (figure 7).

Among all food-restricted groups, there was a main effect of activity on food intake ($F_{(1,54)}$ = 19.710, $p < 0.001$, Table 6 (B)), although it did not rise to a significant level following the post hoc test. Among all female food-restricted groups, food restriction in the presence of a neonatal separation showed a substantial effect: MSFDIET, but not FDIET, group ate a significant higher amount of food on day 1, p = 0.022, compared to both ABA female groups (figure 7). A similar observation was made in males, i.e. MSMDIET compared to MSMABA and MABA on day 1, p = 0.011 (figure 7).

No significant between-group difference from the neonatal treatment or sex was observed among the ABA subjects. However, along the experimental days, each group significantly increased the amount of food ingested, p < 0.001, p < 0.001, p < 0.001, independently of the neonatal treatment (figure 7).

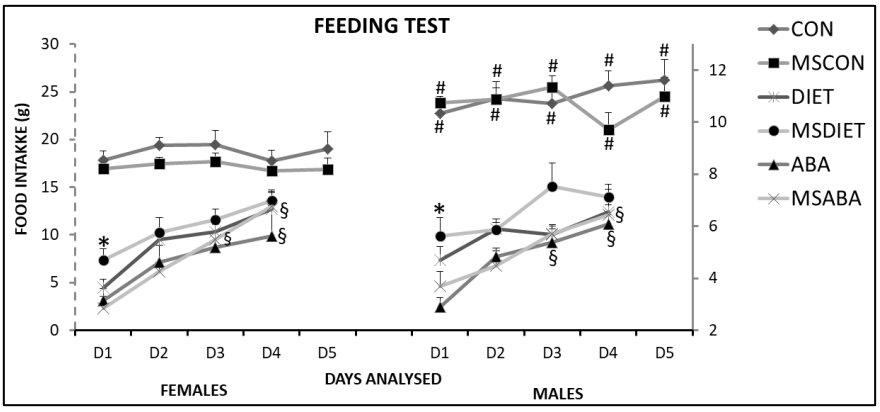


Figure 7: Data on food ingested in the course of the ABA protocol. The gridlines represent data for control groups and solid line represent data for all food-restricted groups. The graph shows the variations of the amount of food intake (in grams) considering, separately, females and males.
The means and the standard error of the mean (SEM) are represented in the graphs.
Asterisks (*) indicate significant differences (p < .05) among the differently treated groups (MSFDIET vs. MSFABA & FABA; MSMDIET vs. MSMABA & MABA).
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (activity-based anorexia).

4.3.2 Body weight analysis

There was a main effect of sex ($F_{(1,50)}$ = 23.883, p < 0.001) and treatment ($F_{(3,50)}$ = 37.816, p < 0.001) when all groups were combined, Table 6 (A). Higher amount of food ingested resulted in a significant increase in body weight, p < 0.001, in the control male compared to female groups from the second till the last day of the analysis (figure 8).

Among all food-restricted groups, there was a main effect of sex ($F_{(1,54)}$ = 5.074, p < 0.05) and activity ($F_{(1,54)}$ = 5.538, p < 0.05) on body weight loss, Table 6 (B), but it didn't rise to a significant level after the post hoc analysis. Among females, the group with only food restriction compared to the ABA groups had a significantly reduced bodyweight drop on day 3, p = 0.019, and day 4, p < 0.001 (figure 8). Interestingly, although it did not rise to significance, a higher bodyweight loss was observed in the ABA male non-maternally separated with respect to maternally separated group, (figure 8). The daily within-group analysis In the ABA group showed a significant reduction in body weight on the last day of the protocol compared to day 1 in both maternally separated, p < 0.001, and non-handled, p < 0.001, female groups (figure 8). By observation, the decline in body weight in males, compared to females, was less drastic (figure 8).

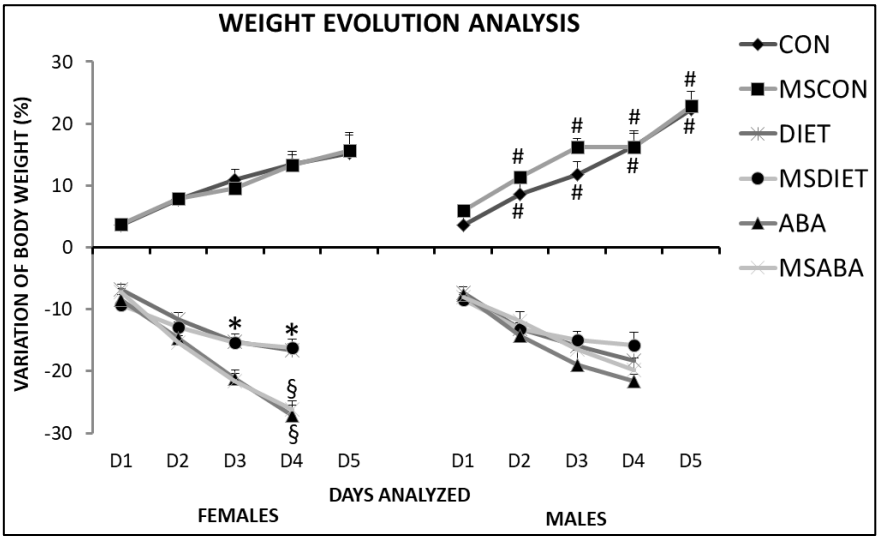


Figure 8: Data on weight evolution during the ABA protocol. The body weight loss (in food-restricted group) or gain (in control group) was calculated as the difference in percentage between the daily body weight and the body weight at the starting date of the ABA protocol. Results are shown separately for females and males.
The means and the standard error of the mean (SEM) are represented in the graphs. Asterisks (*) indicate significant differences (p < .05) among the differently treated groups (FDIET & MSFDIET vs. FABA & MSFABA).
Abbreviation: MS (maternally separated), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted + wheel access).

TABLE 6 (A): Two-way repeated-measures ANOVA main effects on feeding test and body weight data during the ABA protocol for all groups

PARAMETERS	SEX	TREATMENT	SEX*TREATMENT
FEEDING TEST (g)	$F_{(1,78)} = 18.805$, $p < 0.001$	$F_{(5,79)} = 197.227$, $p < 0.001$	$F_{(5,79)} = 7.380$, $p < 0.001$
BODY WEIGHT LOSS (%)	$F_{(1,78)} = 25.183$, $p < 0.001$	$F_{(5,78)} = 31.322$, $p < 0.001$	n.s

Note: The values of *F* and *p* were obtained through a two-way repeated-measures ANOVA, used to analyse the possible effects of sex (M, males, vs. F, females), treatment (CON, DIET, ABA, MSCON, MSDIET, MSABA) and their interactions on parameters collected during the period of the ABA protocol.
n.s (not significant).

TABLE 6 (B): Two-way repeated-measures ANOVA main effects on feeding test and body weight data during the ABA protocol for all food-restricted groups.

PARAMETERS	SEX (M/F)	TREATMENT	SEX*TREATMENT
FEEDING TEST (g)	n.s	$F_{(3,54)} = 7.245$, $p < 0.001$	n.s
BODY WEIGHT LOSS (%)	$F_{(1,54)} = 5.074$, $p < 0.05$	n.s	n.s

Note: The values of *F* and *p* were obtained through a two-way repeated-measures ANOVA, used to analyse the possible effects of sex (M, males, vs. F, females) and treatment (DIET, ABA, MSDIET, MSABA) and their interactions on parameters collected during the period of the ABA protocol.
n.s (not significant).

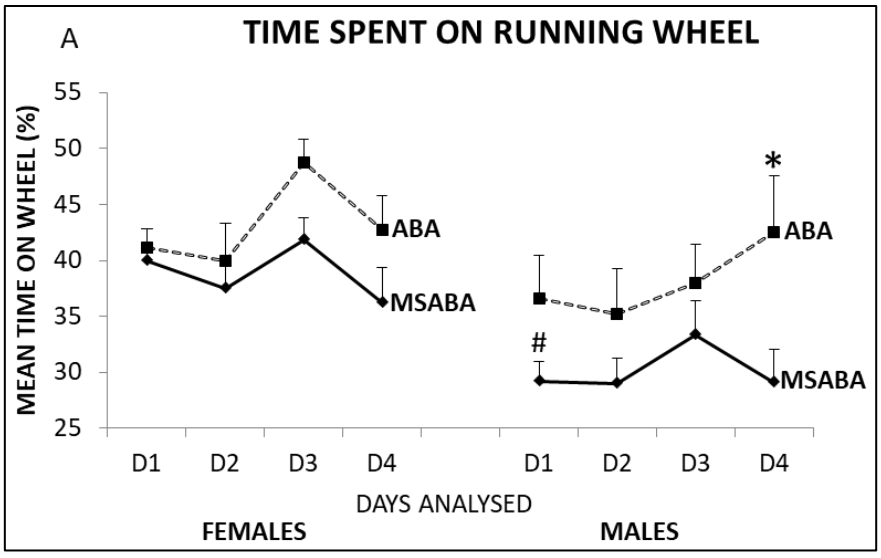
TABLE 6 (C): Two-way repeated-measures ANOVA main effects for single-sex groups on feeding test and body weight data during the ABA protocol

PARAMETER	STRESS (MS/NH)	ACTIVITY (ACT/NO ACT)	STRESS*ACTIVITY
FEEDING TEST (g)			
ALL FEMALE FR	n.s	$F_{(1,29)} = 8.072$, $p < 0.05$	n.s
ALL MALE FR	n.s	$F_{(1,30)} = 12.279$, $p < 0.05$	n.s
BODYWEIGHT LOSS (%)			
ALL FEMALE FR	$F_{(1,29)} = 9.533$, $p < 0.05$	n.s	n.s
ALL MALE FR	n.s	$F_{(1,36)} = 8.864$, $p = 0.05$	n.s

Note: The values of *F* and *p* were obtained through a two-way repeated-measures ANOVA, used to analyse the possible effects of stress MS vs. non-handled and activity and their interactions on parameters collected during the period of the ABA protocol.
n.s (not significant).

4.3.3 Time spent and activity levels on running wheel during ABA protocol

There was a main effect of sex ($F_{(1,25)}$ = 11.072, p < 0.001) and treatment ($F_{(1,25)}$ = 13.128, p < 0.05 see Table 7) of time spent on the wheel, however, significant levels were observed only on day 1 between maternally separated male and female, i.e. MSFABA > MSMABA, p = 0.032, and on the last day between the male groups, i.e. MABA > MSMABA, p = 0.033, figure 9A. In all ABA groups, with the exception of the maternally separated male group, activity levels statistically increased to significant levels across the days analysed, figure 9B. Although it was significant, a decline in time spent and activity on the wheel was observed in both female groups on the final day of the analysis. Comparing sexes, females covered significantly higher distances on the wheel with respect to males in all the first three days of the analysis, figure 9B. A trend of increased activity levels was observed for both non-maternally separated male and female group compared to respective maternally separated groups, figure 9B.



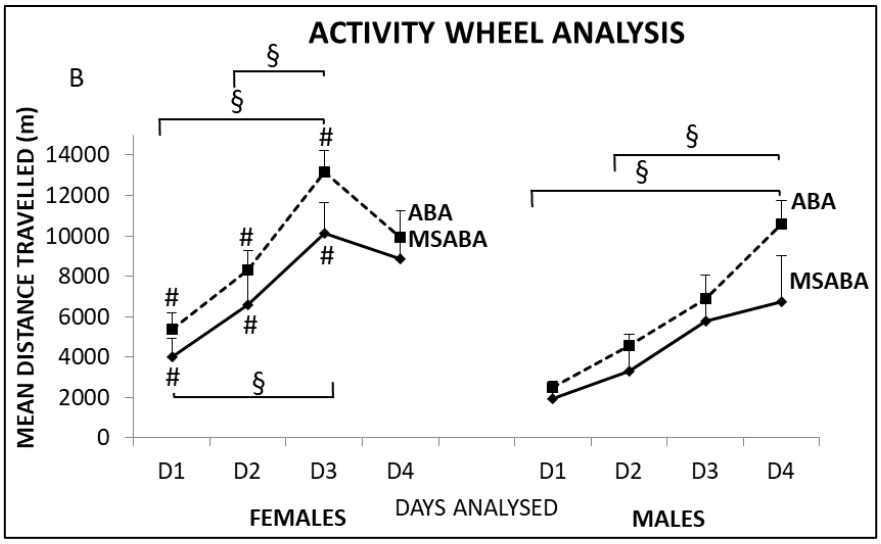


Figure 9: Parameters of physical activity. Graphs show the variations of the time spent to run (expressed in percentages), (**A**), and the distance travelled on running wheel (expressed in meters), (**B**), during the 4 days of ABA protocol, considering, separately, females and males.
The means and the standard error of the mean (SEM) are represented in the graphs.
Asterisks (*) indicate significant differences ($p < .05$) among the differently treated groups (MABA vs. MSMABA)
Number signs (#) indicate sex differences in the same groups ($p < .05$, FABA vs. MABA, MSFABA vs. MSMABA).
Section sign (§) indicates significant differences ($p < .05$) day by day within a group
MS (maternal separation), ABA (activity-based anorexia).

TABLE 7: Two-way repeated-measures ANOVA main effects on wheel analysis during the ABA protocol.

PARAMETER	DAYS	SEX (M/F)	TREATMENT (MS vs NH)	SEX * TREATMENT
TIME SPENT ON WHEEL (%)	n.s	$F_{(1,25)} = 11.072$, $p < 0.001$	$F_{(1,25)} = 13.128$, $p < 0.05$	n.s
WHEEL ACTIVITY (m)	$F_{(3,114)} = 13.970$, $p < 0.001$	$F_{(1,25)} = 16.600$, $p < 0.001$	n.s	n.s

Note: The values of *F* and *p* were obtained through a two-way repeated-measures ANOVA, used to analyse the possible effects of sex (M vs. F for females) and treatment (MS vs. non-handled) and their interactions on parameters collected during the period of the ABA protocol.
Abbreviation: F (females), M (males), MS (maternal separation), NH (non-handled), ABA (activity-based anorexia), n.s (not significant).

4.3.4 Running wheel analysis in different periods

4.3.4.1 Food anticipatory activity period (FAA)

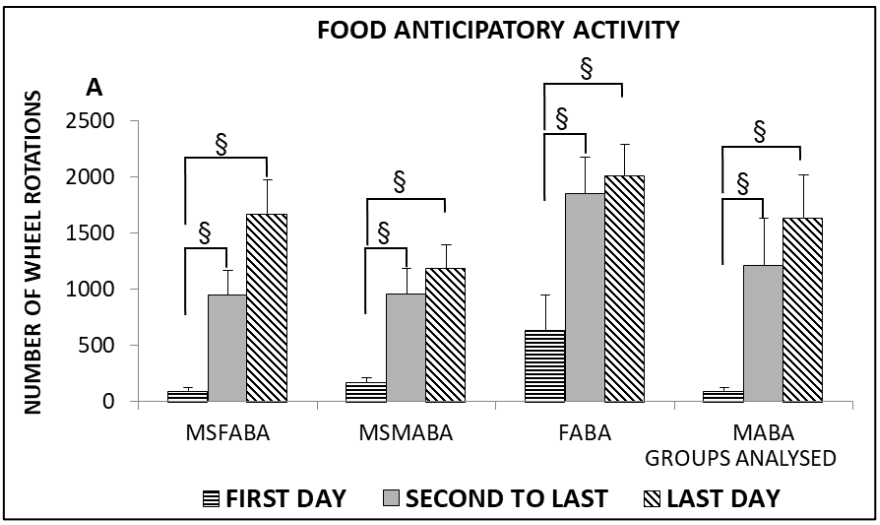
A main effect of days ($F_{(2,74)}$ = 47.392, p < 0.001), sex ($F_{(1,74)}$ = 4.822, p < 0.05) and treatment ($F_{(1,74)}$ = 5.271, p < 0.05) was observed for activity during this period, see **Table 8 A**. Activity levels were increased statistically within each group across the days analysed, figure 10 A.

4.3.4.2 Postprandial activity period (PPA)

Although activity levels on the first day of the analysis was significantly higher in non-handled females compared to males, the progressional increase in wheel rotations across all the days was higher in the male non-handled group. In fact, there was a higher number of rotations for MABA on the last compared to the first day, p = 0.005, figure 10 B**.** Again, we observed a trend of higher activity levels for the most active male group, i.e. MABA, with respect to the most active female group, i.e. FABA, on the second to last and last day of the analysis, figure 6B., figure 10 B.

4.3.4.3 Nocturnal activity period (NA)

The hyperactivity in the non-maternally separated female group was underlined also by wheel analysis during this period (figure 10 C). Contrary to the postprandial period, the progressional activity in this section of the ABA protocol was significantly higher on the last compared to the first day, for the FABA group, p < 0.001, figure 10 C. Although the difference was significant compared only to the non-handled male group, wheel rotations were higher on the second to last and last day in the most active female group, i.e. FABA, compared to all other groups.



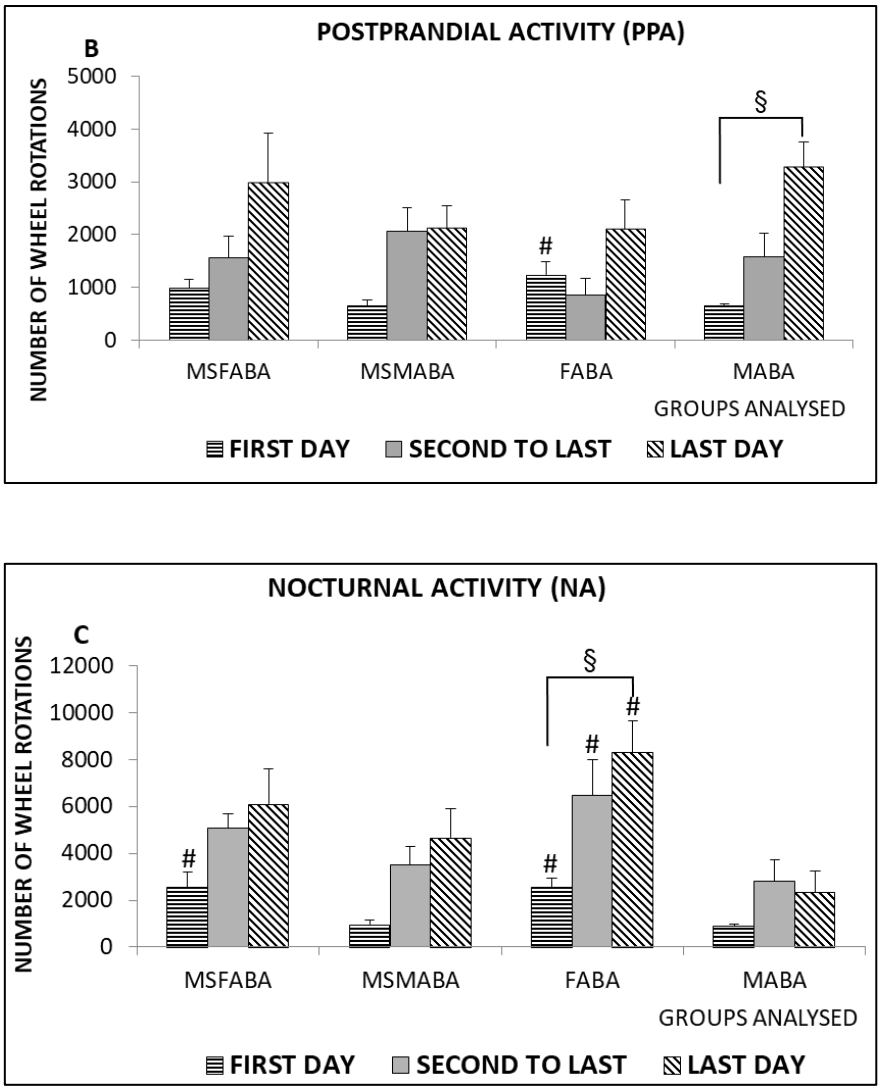


Figure 10: Comparison of FAA, PPA and NA between groups on different days (in terms of before dropout). To avoid bias due to different dropout rates, we considered the first, second to last and last day of the ABA protocol for the analysis. The activity levels prior to food administration are represented in (**A**), the activity levels immediately after food administration in (**B**) and the normal most active period in rodents in (**C**).
The means and the standard error of the mean (SEM) are represented in the graphs.
Asterisks (*) indicate significant differences ($p < .05$) among the differently treated groups (ABA vs. MSABA), number signs (#) indicate sex differences in the same groups ($p < .05$, FABA vs. MABA, MSFABA vs. MSMABA) and section sigh (§) represent the daily differences in the same group.
MS (maternal separation), F (females), M (males), ABA (activity-based anorexia).

TABLE 8 (A): Two-way ANOVA main effects on wheel analysis: within group day by day analysis

PARAMETER	DAYS	SEX (M/F)	STRESS (MS vs NH)	SEX * STRESS
FAA	$F_{(2,74)} = 47.392$, $p < 0.001$	$F_{(1,74)} = 4.822$, $p < 0.05$	$F_{(1,74)} = 5.271$, $p < 0.05$	n.s
PPA	$F_{(2,73)} = 10.899$, $p < 0.001$	n.s	n.s	n.s
NA	$F_{(2,75)} = 12.457$, $p < 0.001$	$F_{(1,75)} = 24.150$, $p < 0.001$	n.s	n.s

TABLE 8 (B): Two-way ANOVA main effects on wheel analysis during different periods of the ABA protocol.

PARAMETER	SEX (M/F)	STRESS (MS vs NH)	SEX * STRESS
FAA (all days)	n.s	n.s	n.s
PPA_FD	$F_{(1,25)} = 7.789$, $p < 0.05$	n.s	n.s
PPA_STL	n.s	n.s	n.s
PPA_LD	n.s	n.s	n.s
NA_FD	$F_{(1,24)} = 21.740$, $p < 0.001$	n.s	n.s
NA_STL	$F_{(1,26)} = 7.101$, $p < 0.05$	n.s	n.s
NA_LD	$F_{(1,25)} = 7.759$, $p < 0.05$	n.s	n.s

Note: The values of *F* and *p* were obtained through a two-way ANOVA, used to analyse the possible effects of sex (M vs. F for females) and stress (MS vs. non-handled) and their interactions on parameters collected during the period of the ABA protocol.

F (females), M (males), MS (maternal separation), NH (non-handled), FAA (food anticipatory activity), PPA (postprandial activity), NA (nocturnal activity), FD (first day), STL (second to last), LD (last day), n.s (not significant).

4.4 DISCUSSION

The results obtained from our analysis showed that in females, the food restriction due to the ABA protocol led to increased locomotor activity without changes in food intake and a drastic body weight loss. In males, compared to females, we observed a decreased physical activity without changes in food intake and a trend of mild drop in body weight, suggesting higher female susceptibility to the protocol. The increased physical activity in females was observed even under ad lib feeding during the wheel pre-exposure period, as reported in Chapter 3, paragraph 3.3.5. The sex-dependent difference in running wheel activity was conferred by a higher food anticipatory activity (FAA) and nocturnal activity (NA) in females and FAA and postprandial activity (PPA) in males. Considering the neonatal treatment, reduced vulnerability to the ABA protocol was observed when subjects were maternally separated, although this was a trend in most cases. As a vital component of the ABA protocol, food restriction alone, i.e. DIET group, also induced a drop in body weight, but minimal compared to the ABA group, especially in females. However, food ingested was significantly higher on day 1 (although there was a trend on days 2 and 3) in the maternally separated DIET compared to the ABA group of both sexes.

A widely held view in the literature is the adverse effects of a prolonged maternal separation on the development of animals due to an observed increase in stress response and fearfulness to novelty in adult life (Caldji et al., 2000; Kalinichev et al., 2002; Pryce & Feldon, 2003). Other reports, however, have indicated a protective effect in adult rats following either brief or long maternally separated protocols (Carrera et al., 2006 & 2009). Implementing the ABA procedure in our analysis, revealed a trend of reduced activity levels and increased food ingestion in both male and female maternally separated groups, thus contradicting earlier reports of poor outcomes in long-maternally separated animals. Studies have shown that the neuroendocrine effect of prolonged early maternal separation is reversed when subjects are exposed to harsh and chronic situations in adulthood. Agreeably, Farinetti et al., 2020, analysing the effect of early life stress combined with the mild-ABA protocol, indicated a reduction in anxiety-related behaviours in maternally separated compared to non-handled female groups (Farinetti et al., 2020). The discrepancy to the effects of the ABA protocol in the presence of the neonatal treatment across different laboratories may hinge on several factors, including age, sex, type of neonatal separation, variability in the ABA protocol and more importantly the test situations used to analyse the impact of the neonatal separation.

The pathological body weight loss in both human and animal AN stem from the effect of heightened activity levels on caloric depletion (Wu et al., 2014). The evolution of the anorexic phenotype in the ABA groups, as indicated, was influenced by activity in all periods of the protocol. However, in the most active female group, FABA, we recorded higher activity levels during the FAA and NA levels. In males, we observed increased activity levels during the FAA and PPA in the most active group, MABA. Among males, activity and time spent on the running wheel were higher, reaching a significant level on the last day, i.e. the time spent, for the non-handled compared to the maternally separated group. This difference corresponded with activity levels during the FAA and PPA but not the NA period. In fact, there was a decline in the levels run across days in the NA period for the MABA group. Similarly, the increased activity in the FABA group was not observed during the PPA period. Surprisingly, there was a trend of lower activity levels on the second to last for the FABA compared to all the other ABA groups and on the last day compared to MSFABA and MABA groups.

We underline in this work the importance of the activity levels prior to food administration, i.e. the FAA, as it occurs in both highly susceptible groups. A significant leap during this activity period was observed in all groups across all the days analysed in the ABA protocol. Agreeably, activity in this period remains a central feature of hyperactivity in this rodent model of anorexia nervosa: the higher the FAA, the more susceptible to the ABA protocol (Hillebrand et al., 2005; Wu et al., 2014; Klenotich et al., 2012). Antipsychotic drugs, Olanzapine and Fluoxetine hydrochloride, observed to reduce the anorexic effect due to their impact on activity levels and body weight were found to reduce activity levels in the FAA period (Klenotich et al., 2012). Furthermore, running wheel activity in other periods synergises to contribute to the drastic weight loss in the ABA protocol. In agreement, Wu et al., 2014 indicated a higher PPA, but not FAA, in the highly susceptible ABA group (Wu et al., 2014), although their experiment was done in only female Wistar rats; in our experiment we employed a neonatal separation in addition to the ABA protocol and compared Sprague Dawley rats of both sexes. These essential differences could explain why we observed a PPA impact in males but a NA in females.

The fact that activity levels in the FAA period, which lasts for only 2-3 hours, is very crucial to the progression of the anorexic phenotype, although in combination with other periods in most cases, indicates that intensity of activity is significant in contributing to the hyperactivity levels observed in AN subjects. As a translational approach, this could be a key measure to consider with respect

to therapies aimed at ameliorating activity levels and the consequential bodyweight loss in anorexic patients since activity in itself is essential for physical wellbeing.

CHAPTER 5: BEHAVIOURAL ALTERATIONS FOLLOWING ABA PROTOCOL INDUCTION IN THE PRESENCE OF MATERNAL SEPARATION

5.1 INTRODUCTION AND AIM OF EXPERIMENT

The efficacy of pharmacological agents to ameliorate anxiety in rodents is assessed primarily using the elevated plus maze (EPM) test (Walf & Frye, 2007). The test is based on the conflict between the rodents' explorative drive and innate fear of open and exposed areas. Thus, an anxious behaviour is depicted by higher entry and more time spent in the closed arms, whereas higher frequency and longer time in the open arms indicate anxiolytic behaviour (Walf & Frye, 2007; Farinetti et al., 2020). A significant advantage in using this paradigm is the absence of noxious stimuli that generally cause a conditioned response (Walf & Frye, 2007), thus avoiding bias in investigating the effect of drugs, hormones or protocol induced (Walf & Frye, 2007). In addition, specific brain regions known to modulate anxiety has been analysed using the EPM apparatus. For instance, using this apparatus, the anxiolytic effects of benzodiazepine receptor agonist and antagonist on the DRN were elucidated (Gonzalez & File, 1997).

As one of the most widely employed tools in animal psychology, the open field (OF) is used to evaluate the general locomotor behaviour of the tested animal but can also be used to test for anxiety; thus, remaining close to the walls is a sign of major anxiety-like behaviour and vice versa (Walsh & Cummins, 1978; Seibenhener & Wooten, 2015). Although conclusions on the psychological and physiological concepts underlying the OF test are straightforward, it is recommended the comparison with other tests (Seibenhener & Wooten, 2015).

Combining both tests described, we analysed the changes in behaviour with respect to exploratory and anxiety following the implementation of maternal separation, food restriction and ABA or in their combinations in the subjects.

5.2 MATERIALS AND METHODS

5.2.1 Animals

See Chapter 4, paragraph 4.2.2, figure 6.

5.2.2 Behavioural analysis

All groups, i.e. ABA, DIET and CONTROL, were submitted to two different behavioural tests to evaluate their anxiety and locomotor/exploratory activity: the OF and EPM. The criterion used for the analysis was as follows:

- ABA group: 20-25% loss of bodyweight
- DIET group: 15-20% loss of bodyweight
- CONTROL group: analysed together with respective ABA and DIET group

All behavioural tests, beginning with the open-field test, were performed in the afternoon, starting from 2:00 p.m. (during the light cycle). Before the actual onset of the behavioural test, animals were left in the room with dim lighting for 2 hr to acclimate to the dark conditions. Due to the rapid loss of body weight in ABA and DIET groups, both behavioural tests were performed on the same day with an hour interval between the tests for each animal.

5.2.2.1 OF

The arena was a square measuring 50 × 50 cm, with 40-cm-high walls; the centre of the arena was defined as a square equidistant (11.5 cm) from all four walls. The apparatus was placed inside a dark testing room, illuminated only by a lamp pointing toward the ceiling. This test is based on fear of open spaces; remaining close to the walls is a sign of central anxiety-like behaviour. Moreover, the OF is used to evaluate the hyperactivity of the tested animal (Kim et al., 2012; Sagvolden, Hendley, & Knardahl, 1992). Rats were individually placed in the same corner of the apparatus and allowed to explore freely for 5 minutes (van Zyl, Dimatelis, & Russell, 2016). The activity of each rat was recorded with a digital camera placed above the apparatus, and the resulting video was analysed offline in EthoVision 8 Software (Noldus Information Technology; Noldus, Spink, & Tegelenbosch, 2001) by a researcher blinded to the treatments. The following parameters were collected:

- the total distance travelled in the arena
- the distance travelled in border

- the number of entries in the border of the arena,
- the time spent in the centre
- the frequency of rearing and grooming behaviour

In addition to the analysis with the Ethovision software, we registered how often animals show the behaviour of rearing and grooming in the centre of the arena. *Rearing* was defined as the number of times that an animal stood up on its hind limbs in an upright position.

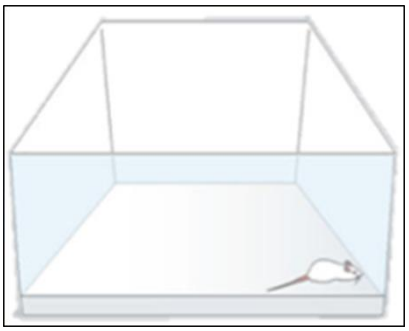


Figure 11: OF apparatus used for behavioural analysis.
OF (open field)

5.2.2.2 EPM

The EPM apparatus was elevated 50 cm from the ground and consisted of two arms (each 50 × 10 cm) enclosed by 30-cm-high sides and end walls (closed arms) and two other arms (each 50 × 10 cm) with no walls (open arms). At the centre of the apparatus, there was also a square platform measuring 10 × 10 cm. The apparatus was placed inside a dark testing room, illuminated by a lamp pointing toward the ceiling, to illuminate the closed and open arms equally. This test is based on the conflict between the rat's exploratory drive and its innate fear of exposed areas; thus, less time spent exploring the open arms indicates greater anxiety-related behaviour. At the beginning of the test, each rat was placed on the central platform, facing an open arm, and was allowed to explore the plus maze freely for 5 min (Noldus et al., 2001; Walf & Frye, 2007; van Zyl et al., 2016). The rat's activity was recorded with a digital camera and analysed using EthoVision 8 Software).

The parameters evaluated for this test include:

- the total distance travelled in the maze
- the number of entries into arms
- the time spent in the arms and centre

In addition, we analysed how many times animals show the behaviour of open head dip, which was defined as the number of times the rat looked over the edge of the maze while its body was entirely in the open sections.

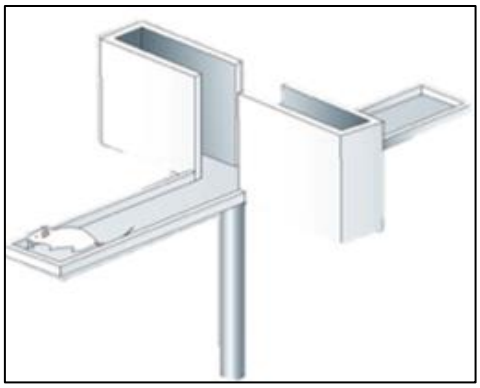


Figure 12: EPM apparatus used for behavioural analysis.
EPM (elevated plus maze)

5.2.3 Statistical analysis

The data collected during the behavioural test were analysed by a two-way ANOVA: sex, treatment and their interactions. For single sex analysis, a three-way ANOVA was used to analysed the effect of stress (MS/NH), activity (ACT/NO ACT) and food intake (FR/NO FR) and their interactions. All analyses were performed after verifying the normality of the data with the Shapiro test and were followed, if the main effects were significant, by the Student-Newman-Keuls (SNK) post hoc test. The SPSS 24.0 program was used to calculate the *p* values, and the significance threshold was set at $p < 0.05$.

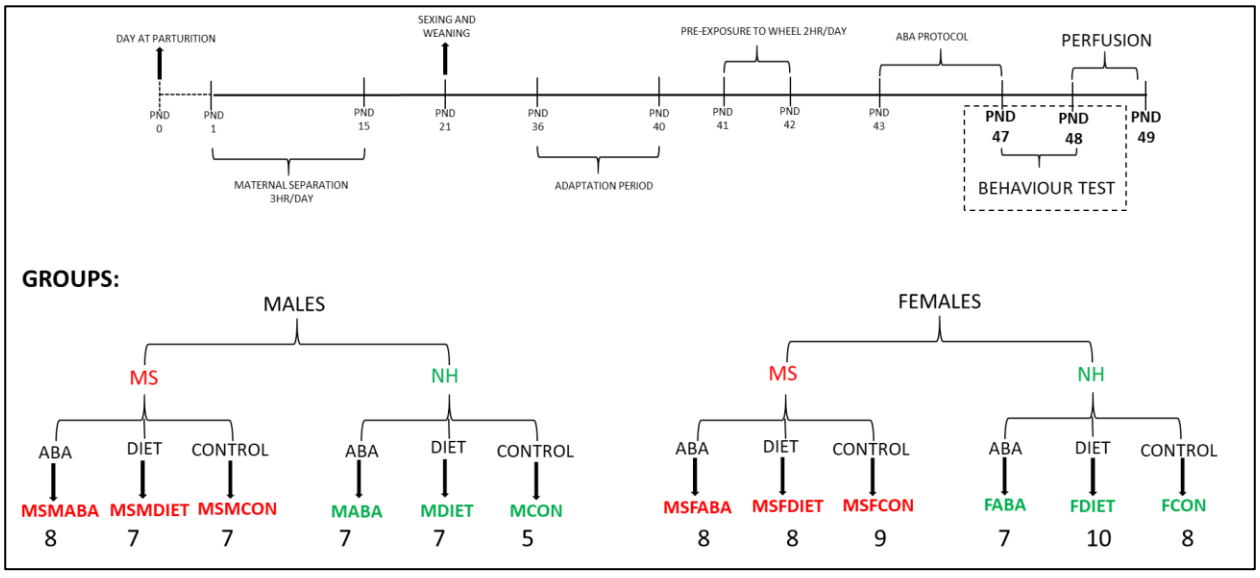


Figure 13. Summary of experimental protocol and groups analysed during the behaviour test.
We performed both an open field and elevated plus maze test for each group to analyse exploratory and anxiety behaviour.
PND (postnatal day), MS (maternal separation), NH (non-handled), ABA (activity wheel with food restriction), DIET (food restriction without activity wheels), CONTROL (ad lib. feeding and without wheel access).

5.3 RESULTS

5.3.1 Open field test

5.3.1.1 Distance travelled in the arena

The ANOVA analysis for the total distance travelled in the arena when all groups were combined revealed a main effect on treatment ($F_{(5,79)}$ = 6.90, p < 0.001, Table 9) but not on sex nor sex and treatment interaction.

Among females, a two-way ANOVA revealed a main effect of stress ($F_{(1,40)}$ = 9.766, p < 0.01), activity ($F_{(1,40)}$ = 13.200, p < 0.01) and the interaction between them ($F_{(1,40)}$ = 5.793, p < 0.05, Table 10). Following the SNK post hoc test, among the group with neonatal separation, we observed a significant reduction in distance covered when subjects with activity (MSFABA) were compared with those without activity and food restriction (MSFCON) and only food-restricted group (MSFDIET), p = 0.001 (figure 14). Among the ABA group, subjects without neonatal separation, FABA, covered a higher distance than the MSFABA group, p = 0.001 (figure 14).

The main effect of activity ($F_{(1,33)}$ = 13.405, p < 0.01) and interaction between activity and stress ($F_{(1,33)}$ = 15.529, p < 0.01, Table 11) was observed among males. Similarly, there was a reduced distance travelled for males with activity compared to those without activity independently of the neonatal separation, that is, MABA versus MCON & MDIET, p = 0.001 and MSMABA versus MSMCON, p = 0.001 (figure 14). A difference in the distance covered was also observed between MSMDIET and MSMCON, p = 0.001 (figure 14). Among groups with only diet restriction, neonatal separation reduced the distance travelled in the arena, p = 0.001 (figure 14).

Comparing sexes, in the presence of a neonatal separation and only food restriction, females covered a greater distance than males, i.e. MSFDIET versus MSMDIET, p = 0.002 (figure 14).

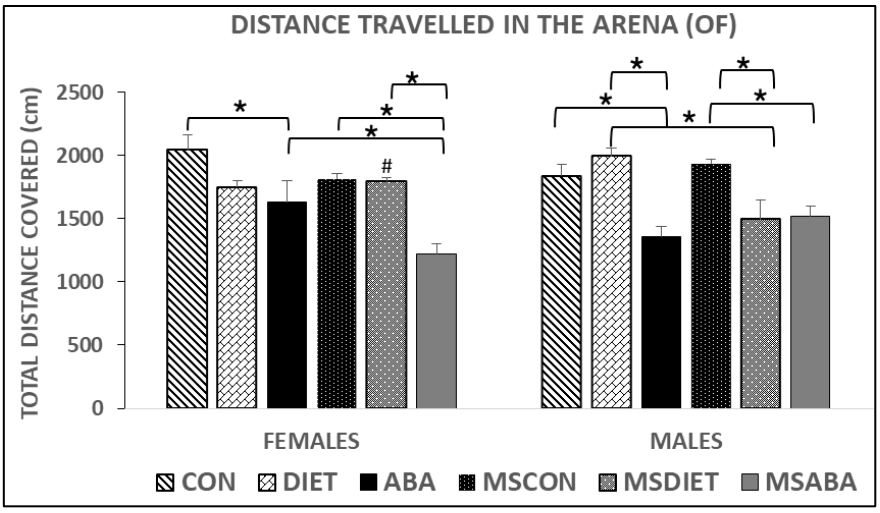


Figure 14: The histograms show the results for the total distance covered in the open field (expressed in centimetres). The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences ($p < .05$) among the differently treated groups (e.g. MCON vs. MABA) and number signs (#) indicate significant sex differences ($p < .05$) in the same group (e.g. MSFDIET vs. MSMDIET).
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.1.2 Distance travelled in the border

The ANOVA analysis for the distance travelled in the border when all groups were combined revealed a main effect of treatment ($F_{(5,79)}$ =6.96, $p < 0.001$, Table 9) but not of sex nor sex and treatment interaction.

Among females, a two-way ANOVA revealed a main effect of activity ($F_{(1,41)}$ = 10.502, $p < 0.01$, Table 10). As shown in figure 15, the ABA protocol reduced the distance travelled close to the walls of the apparatus with a significant effect in those without the neonatal treatment, i.e. FABA versus FCON, $p = 0.001$.

As observed in the males, there was a reduced distance in the border for MABA compared to MCON and MDIET, $p = 0.001$ (figure 15). Among male groups with only food restriction, the maternal separation procedure reduced the distance travelled in the border of the arena, $p = 0.001$ (figure 15).

Comparing only food-restricted male and female groups, the neonatal separation reduced the distance travelled in the border in males, i.e. MSFDIET versus MSMDIET, $p = 0.001$ (figure 15). In contrast, in the absence of the separation protocol, the distance was increased in males, i.e. FDIET versus MDIET, $p = 0.001$ (figure 15).

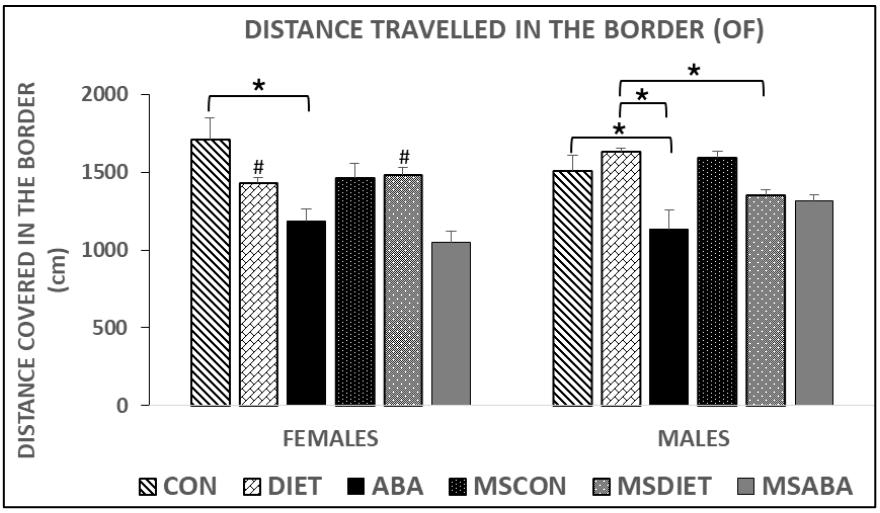


Figure 15: The histograms show the results for the distance covered close to the walls in the open field (expressed in centimetres). The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences ($p < .05$) among the differently treated groups (e.g. MCON vs. MABA) and number signs (#) indicate significant sex differences ($p < .05$) in the same group (e.g. MSFDIET vs. MSMDIET).
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.1.3 Frequency in the border

Among females, a two-way ANOVA revealed a main effect of stress ($F_{(1,42)} = 6.008$, $p < 0.05$) and activity ($F_{(1,42)} = 10.020$, $p < 0.01$, Table 10). As a whole, ABA protocol independently of the neonatal separation reduced the number of entries in the border. However, following the Student Newman-Keuls (SNK) test, we observed a significant level only in MSFABA compared to MSFCON and MSFDIET groups, $p = 0.001$ (figure 16). In addition, among the female ABA group, the absence of the separation protocol increased the number of visits to the border, $p = 0.001$ (figure 16).

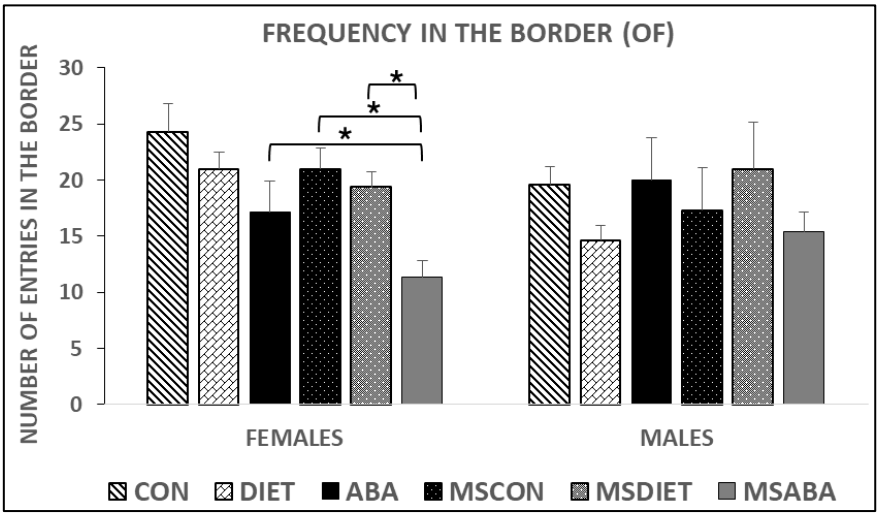


Figure 16: The histograms show the results for the number of times each group visited the border of the open field. The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences ($p < .05$) among the differently treated groups (e.g. MABA vs. MSMABA)
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.1.4 Duration in the centre

The two-way ANOVA indicated a main effect of stress ($F_{(1,33)}$ = 5.308, p < 0.01, Table 11) among males. Following the SNK post hoc analysis, a significantly higher time was spent by male maternally separated controls, i.e. MSMCON, to controls that were non-handled, i.e. MCON, p = 0.007 (figure 17). Comparing sexes, we also observed a higher duration spent for MSMCON compared to MSFCON, p = 0.007 (figure 17).

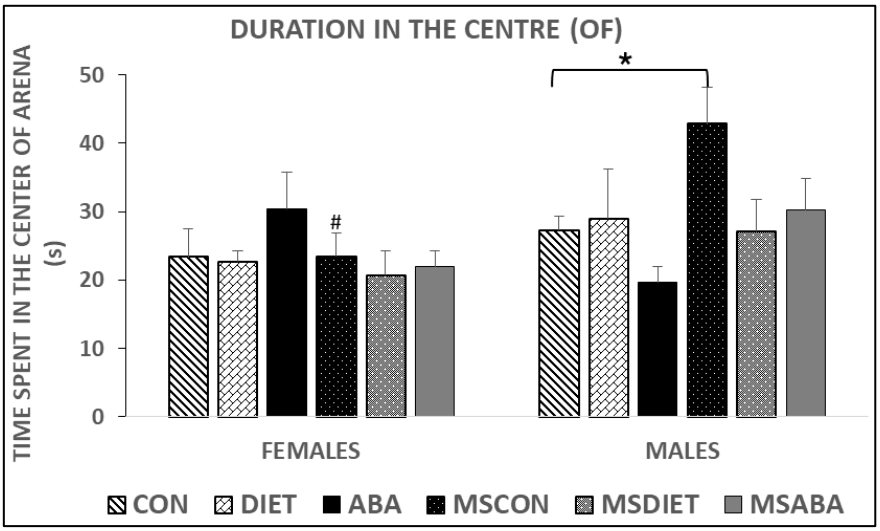


Figure 17: The histograms show the results for the time spent in the centre of the open field (expressed in seconds). The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences (p < .05) among the differently treated groups (e.g. MCON vs. MSMCON) and number signs (#) indicate significant sex differences (p < .05) in the same group (e.g. MSFCON vs. MSMCON).
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.1.5 Frequency of rearing

When all groups were combined, the ANOVA analysis revealed a main effect of treatment ($F_{(5,79)}$ =2.57, p = 0.033, Table 9) but not of sex or treatment and sex interaction.

Comparing only females, a two-way ANOVA revealed a main effect of stress ($F_{(1,42)}$ = 4.561, p < 0.05, Table 10). Accordingly, we observed that this anxiolytic behaviour was significantly higher for female controls without maternal separation, i.e. FCON, than controls with the neonatal treatment, i.e. MSFCON, p = 0.009 (figure 18). Considering only the diet-restricted group, the neonatal treatment significantly reduced the frequency of this behaviour, i.e. MSFDIET > FDIET, p = 0.0427 (figure 18). Lastly, maternally separated with ABA protocol reduced the expression of this behaviour compared to non-maternally separated group, i.e. MSFABA < FABA, p = 0.042 (figure 18).

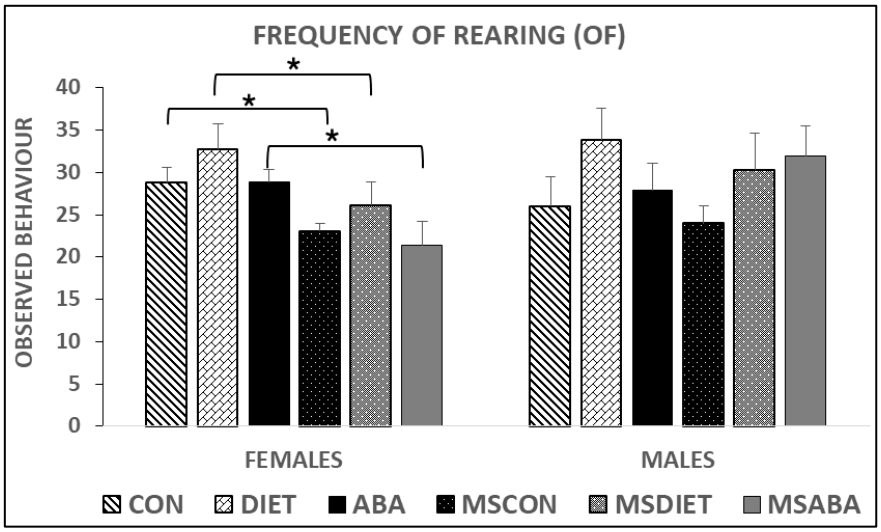


Figure 18: The histograms show the results for the number of times a rearing behaviour was expressed in the open field. The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences (p < .05) among the differently treated groups (e.g. FABA vs. MSFABA)
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.1.6 Frequency of grooming

Correspondingly, the higher frequency of rearing in most non-handled groups was contrasted by a trend of reduced grooming frequency in females (figure 19). In fact, among females, the ANOVA test indicated a main effect of stress for this parameter ($F_{(1,40)}$ = 4.509, p < 0.05, Table 10). The post hoc analysis revealed a higher grooming behaviour expression in the MSFABA than in the FABA group, p = 0.029 (figure 19).

Although there were not significant differences among males, there seemed to be a trend of reduced grooming frequency in all the maternally separated compared to respective non-handled groups (figure 19).

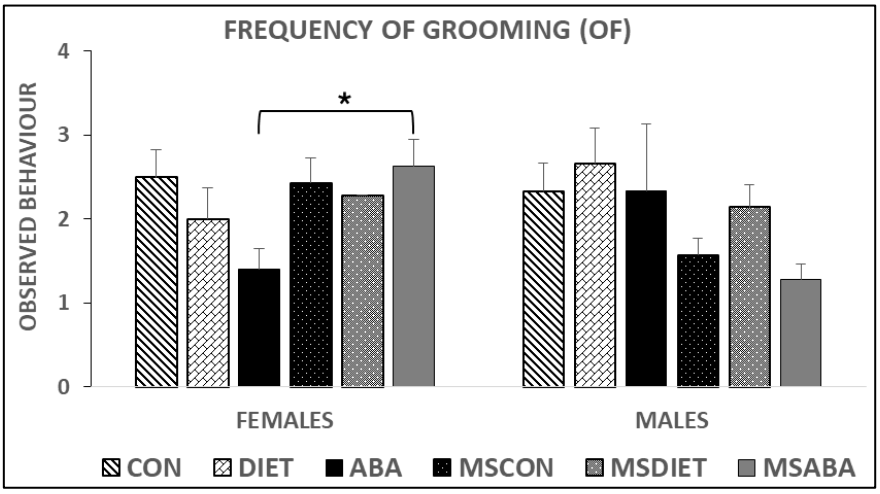


Figure 19: The histograms show the results for the number of times a grooming behaviour was expressed in the open field. The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences (p < .05) among the differently treated groups (e.g. FABA vs. MSFABA)
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.2 Elevated plus maze (EPM)

5.3.2.1 Total distance travelled

The ANOVA analysis for the total distance travelled combining all groups revealed a main effect on the interaction between sex and treatment ($F_{(5,76)}$ = 2.81, p = 0.022, Table 9).

Focusing on only females, a two-way ANOVA showed a main effect of activity ($F_{(1,43)}$ = 4.563, p < 0.05, Table 10), with a significant level when FABA and MSFABA were compared with FCON, p = 0.008 (figure 20). Independently of the ABA protocol and maternal separation, we also observed a significant increase in the total distance travelled for males compared to females, p = 0.046 (figure 20).

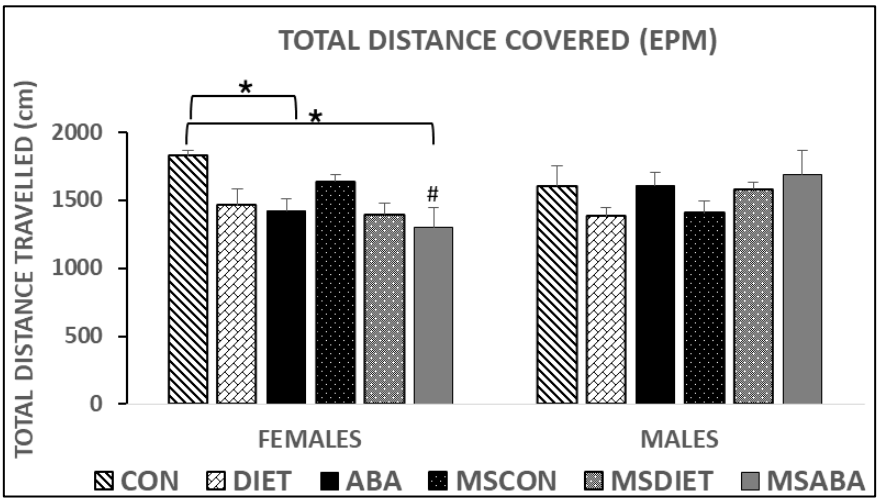


Figure 20: The histograms show the results for the total distance covered in the EPM apparatus (expressed in centimetres). The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences (p < .05) among the differently treated groups (e.g. FCON vs. FABA) and number signs (#) indicate significant sex differences (p < .05) in the same group (e.g. MSFABA vs. MSMABA).
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.2.2 Frequency of entry in the closed arms

The statistical analysis on the number of times a group visited these arms indicated a main effect of sex ($F_{(1,78)}$ = 5.11, p = 0.027, Table 9).

A reduced number of entries into the closed arms were observed when female groups were either food-restricted or given activity wheels independently of the maternal separation protocol (figure 21). Statistically, among female non-maternally separated groups, a significant decline in the frequency of entry was observed for the ABA group compared to FCON p = 0.015 (figure 21).

A similar indication was made in males; that is, a trend of reduced number of visit in these arms was observed in DIET and ABA group independently of the neonatal treatment (figure 21).

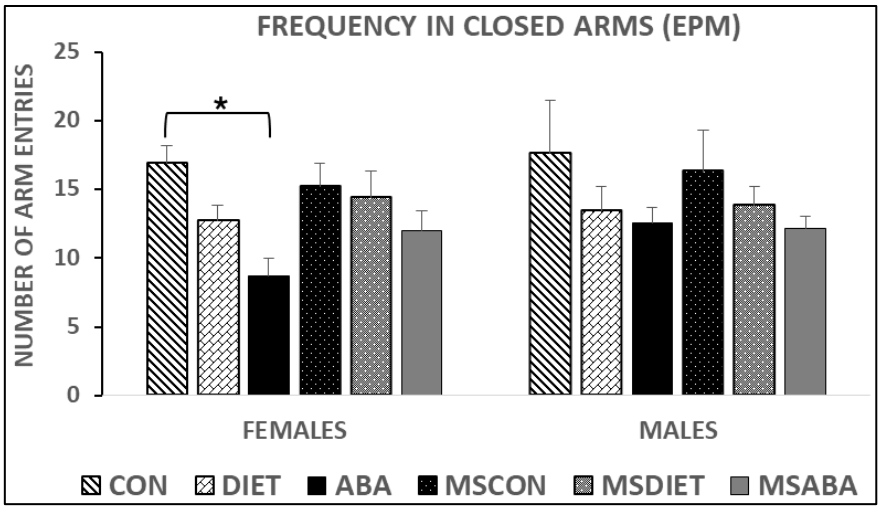


Figure 21: The histograms show the results for the number of times a group visited the closed arms of the EPM. The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences ($p < .05$) among the differently treated groups (e.g. FCON vs. FABA)
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.2.3 Frequency of entry in the open arms

Combining all groups, a main effect of treatments ($F_{(5,77)} = 3.96$, $p = 0.003$, Table 9) was observed. As opposed to the entry in the closed arms, frequency in the open arms, per observation, revealed a higher incidence in groups receiving food restriction or when coupled with wheel access, especially in males (figure 22). Comparing control group with maternal separation, males recorded a significantly higher number of entries in the open arms, $p = 0.053$ (figure 22).

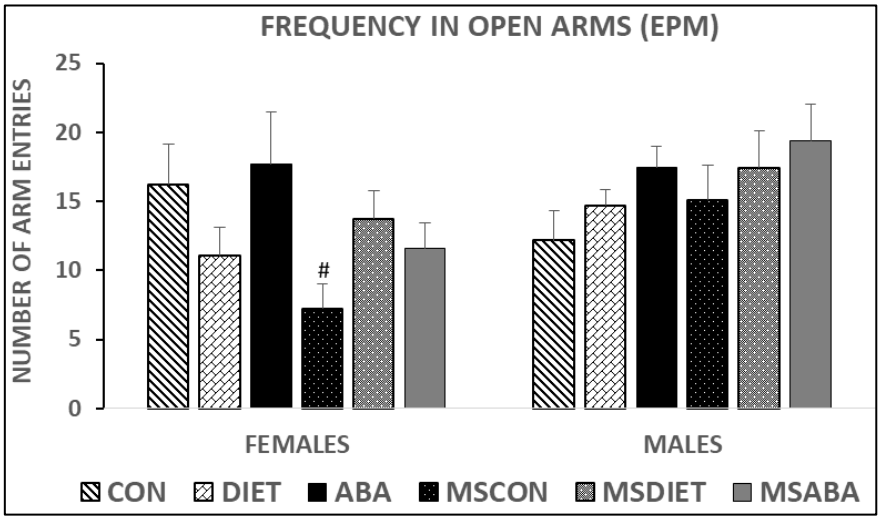


Figure 22: The histograms show the results for the number of times a group visited the open arms in the EPM apparatus. The mean and the standard error of the mean (SEM) are represented in the graph.
Number signs (#) indicate significant sex differences ($p < .05$) in the same group (e.g. MSFCON vs. MSMCON).
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.2.4 Duration in the closed arms

For this parameter, the two way ANOVA revealed a main effect of treatment ($F_{(5,77)}$ = 3.78, p = 0.004, Table 9). Treatment arising from food restriction or coupled with activity reduced the time spent in the closed arms in both sexes. In fact, the post hoc analysis revealed a reduced time spent for FABA compared to FCON, p = 0.040, and MSMABA compared to MSMCON, p = 0.032 (figure 23). In addition, a statistical difference for this parameter was observed when we compared MSFABA with MSMABA, p = 0.023 (figure 23).

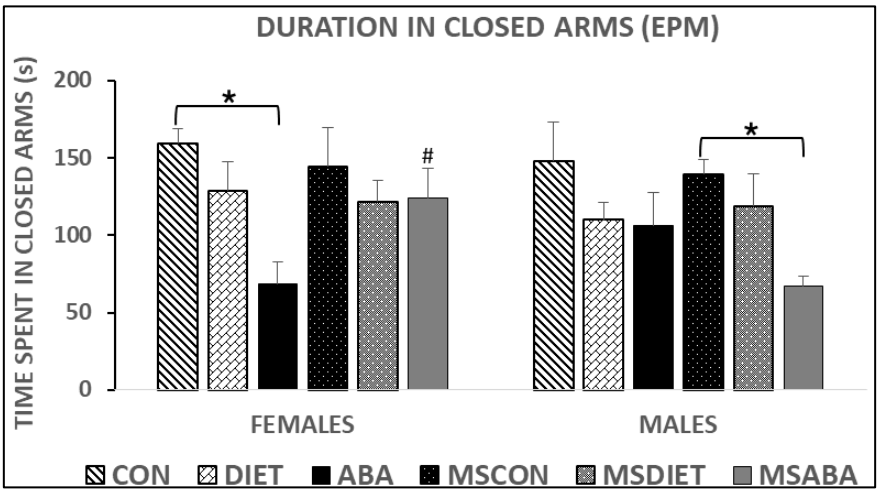


Figure 23: The histograms show the results for the time spent in the closed arms of the EPM apparatus. The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences (p < .05) among the differently treated groups (e.g. FCON vs. FABA) and number signs (#) indicate significant sex differences (p < .05) in the same group (e.g. MSFABA vs. MSMABA).
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.2.5 Duration in the open arms

Expectedly, analysis on time spent in the open arms revealed a contrary result compared with observations made in the time spent in the closed arms.

Again, we observed a main effect of treatment ($F_{(5,79)}$ = 3.56, p = 0.006, Table 9) when all groups were combined. Although a significant difference was seen comparing only MABA with MCON, p = 0.022 (figure 24), a trend of increased duration in open arms was observed for groups either food-restricted or when coupled with activity independently of the maternal separation protocol (figure 24). In fact, in female maternally separated ABA group, a Pearson correlation analysis revealed a significant highly negative relationship between this parameter and both frequency ($r(6)$ = - 0.754, p = 0.031) and time spent ($r(6)$ = -0.868, p = 0.005) in the closed arms of the EMP apparatus.

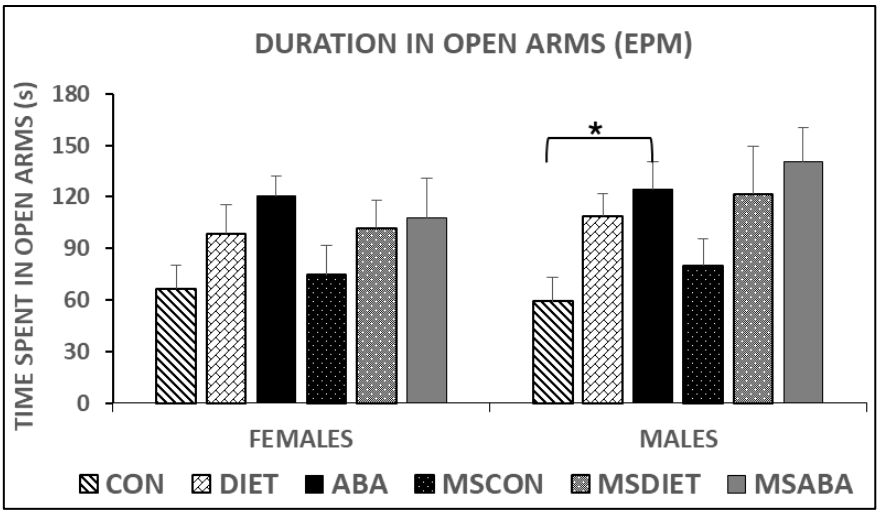


Figure 24: The histograms show the results for the time spent in the open arms of the EPM apparatus (expressed in seconds). The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences ($p < .05$) among the differently treated groups (e.g. MCON vs. MSMCON) and number signs (#) indicate significant sex differences ($p < .05$) in the same group (e.g. MSFCON vs. MSMCON).
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

5.3.2.6 Frequency of head dip

Head dip is a behaviour described as the number of times a subject looked over the edge of the maze while its body was entirely in the open sections. As shown in figure 25, there was a trend of an increased frequency in the expression of this behaviour in male and female groups with wheel access.

Comparing all groups, we observed a main effect of treatment ($F_{(5,78)} = 3.89$, $p = 0.003$, Table 9). A two-way ANOVA indicated a significant increase in the frequency of this parameter for non-maternally separated males with activity, i.e. MABA, compared to the control group, i.e. MCON, $p = 0.022$ (figure 25). The increase in head dip frequency correlated highly positively with the duration in the open arms: $r(5) = 0.797$, $p = 0.032$. Also, there was a very high significant positive correlation between the number of times a head dip behaviour was expressed and the duration in the open arms in both female active groups: MSFABA: $r(6) = 0.916$, $p < 0.001$; FABA: $r(5) = 0.958$, $p < 0.001$.

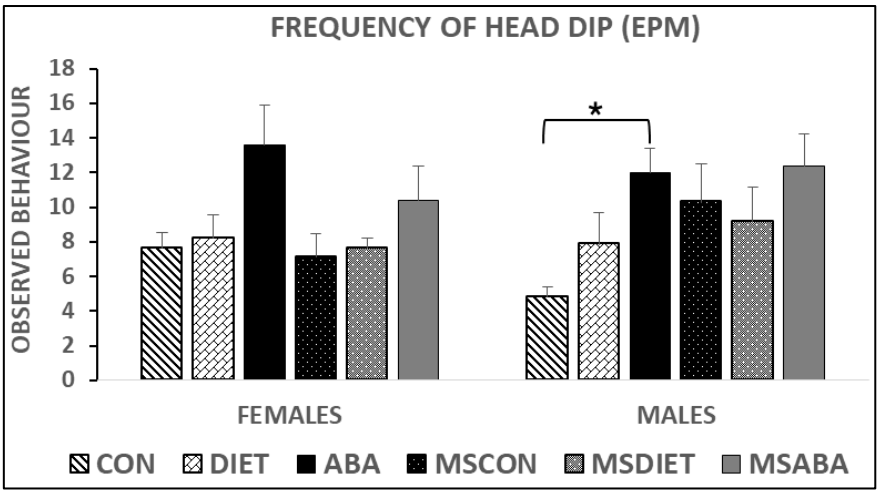


Figure 25: The histograms show the results for the number of times head dip behaviour was expressed in the EPM apparatus. The mean and the standard error of the mean (SEM) are represented in the graph.
Asterisks (*) indicate significant differences ($p < .05$) among the differently treated groups (e.g. MCON vs. MABA)
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

Table 9: Summary of behaviour statistical analysis of the data with main effects for all groups.

PARAMETERS	SEX	TREATMENT	SEX*TREATMENT
OPEN FIELD TEST			
TOTAL DISTANCE TRAVELLED	n.s	$F_{(5,79)}$ =6.90 $p < 0.001$	n.s
DISTANCE TRAVELLED IN THE BORDER	n.s	$F_{(5,79)}$ =6.96 $p < 0.001$	n.s
FREQUENCY OF REARING	n.s	$F_{(5,79)}$ =2.57 $p = 0.033$	n.s
ELEVATED PLUS MAZE TEST			
TOTAL DISTANCE TRAVELLED	n.s	n.s	$F_{(5,76)}$ =2.81 $p = 0.022$
FREQUENCY IN THE CLOSED ARMS	n.s	$F_{(5,77)}$ =3.96 $p = 0.003$	n.s
FREQUENCY IN THE OPEN ARMS	$F_{(1,78)}$ =5.11 $p = 0.027$	n.s	n.s
DURATION IN THE CLOSED ARMS	n.s	$F_{(5,77)}$ =3.78 $p = 0.004$	n.s
DURATION IN THE OPEN ARMS	n.s	$F_{(5,79)}$ =3.56 $p = 0.006$	n.s
FREQUENCY OF HEADDIP	n.s	$F_{(5,78)}$ =3.89 $p = 0.003$	n.s

Note: The values of *F* and *p* were obtained through a two-way ANOVA test, which was used to analyse the possible effects of sex (M, males vs F, females), treatment (CON, DIET, ABA, MSCON, MSDIET, MSABA) and their interaction (sex*treatment) on parameters collected in the behavioural tests.
n.s (not significant).

Table 10: A three-way ANOVA showing main effect for behaviour analysis in females

	FACTORS			INTERACTIONS		
PARAMETER	**1. STRESS (MS vs NH)**	**2. ACTIVITY (ACT vs NO ACT)**	**3. FOOD INTAKE (FR vs NO FR)**	**1*2**	**1*3**	**2*3**
ALL FEMALES						
OPEN FIELD						
TOTAL DISTANCE IN ARENA	$F_{(1,40)} = 9.766$, $p < 0.01$	$F_{(1,40)} = 13.200$, $p < 0.01$	n.s	$F_{(1,40)} = 5.793$, $p < 0.05$	n.s	n.s
DISTANCE IN THE BORDER	*n.s*	$F_{(1,41)} = 10.502$, $p < 0.01$	n.s	n.s	n.s	n.s
DISTANCE IN THE CENTER	$F_{(1,43)} = 5.045$, $p < 0.05$	n.s	n.s	n.s	n.s	n.s
FREQUENCY IN THE BORDER	$F_{(1,42)} = 6.008$, $p < 0.05$	$F_{(1,42)} = 10.020$, $p < 0.01$	n.s	n.s	n.s	n.s
FREQUENCY IN THE CENTER	$F_{(1,44)} = 4.561$, $p < 0.05$	$F_{(1,44)} = 5.907$, $p < 0.05$	n.s	n.s	n.s	n.s
FREQUENCY OF REARING	$F_{(1,42)} = 4.561$, $p < 0.05$	n.s	n.s	n.s	n.s	n.s
FREQUENCY OF GROOMING	$F_{(1,40)} = 4.509$, $p < 0.05$	n.s	n.s	n.s	n.s	n.s
EPM						
TOTAL DISTANCE COVERED	n.s	$F_{(1,43)} = 4.563$, $p < 0.05$	n.s	n.s	n.s	n.s
FREQUENCY IN OPEN ARMS	$F_{(1,45)} = 4.102$, $p < 0.05$	n.s	n.s	n.s	n.s	n.s
FREQUENCY IN CLOSED ARMS	n.s	$F_{(1,43)} = 9.803$, $p < 0.05$	n.s	n.s	n.s	n.s
DURATION IN CLOSED ARMS	n.s	$F_{(1,46)} = 6.679$, $p < 0.05$	n.s	n.s	n.s	n.s

Note: The values of *F* and *p* were obtained through a three-way ANOVA test, which was used to analyse the possible effects of stress (MS/NH), activity (ACT/NO ACT), food intake (FR/NO FR) and their interactions.
n.s (not significant).

Table 11: A three-way ANOVA showing main effect for behaviour analysis in males

PARAMETER	FACTORS			INTERACTIONS		
	1. STRESS (MS vs NH)	**2. ACTIVITY (ACT vs NO ACT)**	**3. FOOD INTAKE (FR vs NO FR)**	**1*2**	**1*3**	**2*3**
ALL MALES OPEN FIELD						
TOTAL DISTANCE IN ARENA	n.s	$F_{(1,33)} = 13.405$, $p < 0.01$	n.s	$F_{(1,33)} = 15.529$, $p < 0.01$	n.s	n.s
DISTANCE IN THE BORDER	n.s	$F_{(1,30)} = 13.679$, $p < 0.01$	n.s	$F_{(1,30)} = 9.756$, $p < 0.01$	n.s	n.s
DURATION IN THE CENTER	$F_{(1,33)} = 5.308$, $p < 0.01$	n.s	n.s	n.s	n.s	n.s
LATENCY TO ENTER CENTER	$F_{(1,36)} = 6.456$, $p < 0.05$	n.s	n.s	$F_{(1,30)} = 5.039$, $p < 0.05$	n.s	n.s
FREQUENCY OF GROOMING	$F_{(1,30)} = 4.760$, $p < 0.05$	n.s	n.s	n.s	n.s	n.s
EPM						
DURATION IN OPEN ARMS	n.s	$F_{(1,46)} = 5.045$, $p < 0.05$	n.s	n.s	n.s	n.s
DURATION IN CLOSED ARMS	n.s	$F_{(1,46)} = 7.710$, $p < 0.05$	n.s	n.s	n.s	n.s
FREQUENCY OF HEAD DIP	n.s	$F_{(1,37)} = 6.819$, $p < 0.05$	n.s	n.s	n.s	n.s

Note: The values of *F* and *p* were obtained through a three-way ANOVA test, which was used to analyse the possible effects of stress (MS/NH), activity (ACT/NO ACT), food intake (FR/NO FR) and their interactions.
n.s (not significant).

5.4 DISCUSSION

Analysis from the OF and EPM tests revealed that: independently of the MS protocol, groups without any treatment were more explorative than ABA and, in a few cases, food-restricted only groups. From the OF test results, among female ABA groups, maternal separation induced a lower explorative activity. Comparing sexes in only food-restricted groups, MS females were more explorative. Summarizing anxiety measures, in the absence of any treatment, MS induced a higher anxiolytic effect in males than females observed by a significant difference in the time spent in the centre of the OF apparatus. The same result was observed when ABA protocol was induced, i.e. MSMABA < MSFABA in the time spent in the closed arms of the EPM. Among male controls, neonatally separated appeared anxiolytic compared to the non-handled group. Both male and females with only food restriction or coupled with activity appeared anxiolytic compared to respective control groups.

As reported in Chapter 1, paragraph 1.4.1, results obtained following MS induction is conflicting between different laboratories (Oomen et al., 2010; Fujimoto et al., 2014). Carrera et al., 2009, indicated that the MS procedure resulted in greater resistance, especially in females (Carrera et al., 2009). In a recent work by Farinetti et al., 2020, anxiety-related behaviours were significantly reduced in female MS ABA compared with a respective non-handled group (Farinetti et al., 2020). In the absence of food restriction or ABA, behavioural parameters that indicated reduced anxiety was higher in male MS subjects compared to the non-handled group. However, an anxiogenic effect was noted among ABA groups in MSFABA compared to FABA and MSMABA. The group that recorded the highest activity levels, i.e. MSFABA, appeared anxiolytic in the analysis of Farinetti et al., 2020. Similarly, the most active female ABA group was the one which appeared anxiolytic, i.e. the FABA group. A higher female to male vulnerability to the ABA protocol is usually observed due to the rapid weight loss and intense activity levels in females. Irrespective of these observations, females have been observed to usually stay longer in the protocol due to their ability to modulate activity levels in a better way compared to males, as observed in our results in Chapter 4, paragraph 4.3.3. Although a higher anxiolytic effect was observed in the MSFABA group in the work of Farinetti et al., 2020, the behaviour analysis in our work indicated that MSFABA were anxiogenic compared to MSMABA. This seeming contradiction can be explained by the differences in the ABA protocol employed: mild-stress ABA protocol by Farinetti and colleagues, 2021, and classical ABA protocol in our experiment.

Exploratory behaviour is underlined by the conflict between surveying novel/dangerous sites and remaining in familiar surroundings (Mällo et al., 2007). An observed strength of the OF apparatus is its analysis of exploratory activity; however, other authors have argued that the OF arena without any enrichment such as tunnels, compartments or runaways, as in the classical OF apparatus, does not elicit exploratory motivation but, suggestively, an escape behaviour (Genaro & Schmidek, 2000). Therefore, higher distance travelled in the arena cannot be interpreted as explorative (Genaro & Schmidek, 2000). Due to the observed positive correlation between low exploratory and enhanced anxiety (Mällo et al., 2007), we argue in favour of Genaro & Schmidek, 2000 that the higher total distance travelled in the arena by the controls compared to ABA groups does not show higher exploratory activity. In fact, analysis of anxiety parameters indicated a higher anxiolytic effect in ABA and DIET than control groups.

The OF and EPM have been instrumental in providing exploratory and anxiety indexes in the ABA model. In light of the behaviour analysis discussed, the MS + ABA is observed to have a dimorphic effect: it reduces anxiety in males but increases anxiety in females. Nevertheless, the extrapolation of results is always ideal when other parameters or tests are compared.

CHAPTER 6: REWARD SYSTEM'S CONTRIBUTION TO THE EXPRESSION OF THE ANOREXIC PHENOTYPE IN ABA RATS

6.1 INTRODUCTION AND AIM OF EXPERIMENT

Many factors have been linked to the intense activity of ABA animals in the presence of drastic bodyweight loss. Weight loss begins typically as a wilful approach meant to burn calories; however, at later stages, many factors become involved, which seem to be outside the individual's will (Gutiérrez et al., 2002). Authors arguing in favour of a thermoregulatory response indicated that the hyperactivity observed compensates for the decline in body temperature generally associated with weight loss (Gutiérrez et al., 2002). Also, the interplay between neurobiological changes and different aspects of running behaviour in animal subjects has been undertaken to highlight which brain areas are particularly vulnerable to caloric-restriction-induced hyperactivity (Chowdury et al., 2015). In the ABA model, as subjects increasingly become hyperactive, food intake is perceived as aversive, whereas activity becomes rewarding even in the face of an intense negative energy state (Sodersten et al., 2016; Holly et al., 2015). This observation has pioneered the analysis of the impact of the reward system and other associated systems, such as the serotonergic, in both human and animal subjects, as reported in Chapters 1.5.1 and 1.5.2.

Recently, Aspesi and colleagues, using a mild-stress form of the ABA protocol, observed higher expression of dopamine $(DA)^{+}$ cells, especially in the VTA of the ABA compared to control groups. When ABA was induced in the presence of maternal separation, DA expression in the VTA was increased but significantly reduced in the SNpc (Aspesi et al., 2021). Clearly, the inclusion of maternal separation does produce a difference.

Using the classical ABA protocol, the aim of this part of the thesis was to analyse the neuroendocrine alteration in the presence of an adverse early environment focusing primarily on the dopaminergic and serotonergic system. We employed an immunohistochemistry analysis to determine the quantitative differences in the number of cells expressing DA and serotonin (5-HT) among the experimental groups to analyse the contribution of these two systems to the observed behaviour data.

6.2 MATERIALS AND METHODS

6.2.1 Animals

See Chapter 4, paragraph 4.2.2, figure 6.

6.2.2 Tissue preparation

The animals were deeply anaesthetized a day after the behaviour analysis, with an intraperitoneal injection of a ketamine (100 mg/kg of body weight) and xylazine (10 mg/kg of body weight). Then, the animals were transcardially perfused with saline followed by 4% paraformaldehyde (PAF). The brains were removed, stored in a freshly prepared PAF solution for two hours at 4 °C and then washed several times in phosphate-buffered saline (PBS). Next, the brains were stored in a 30% sucrose solution in PBS at 4 °C and frozen on dry ice. The brains were serially sectioned along the coronal plane at a thickness of 40 μm. Sections were collected in multiwall dishes filled with an antifreeze solution (30% glycerol, 30% ethylene glycol, 30% deionized water, 10% NaH2PO4 0.0243M and NaOH 0.0175M in aqueous solution) and stored in the freezer at – 20 °C until utilization.

6.2.3 Immunohistochemistry

The obtained series were processed to evaluate dopamine and serotonin cells in the brain using the free-floating immunohistochemistry technique. In particular, to evaluate the density of DA neurons, the sections of the Ventral Tegmental Area (VTA) and Substantia Nigra pars compacta (SNpc) were processed for L-amino acid decarboxylase (AADC); as a lyase enzyme that convert L-dopa to dopamine, AADC is more specific than other enzymes involved in DA's synthesis. For instance, using other enzymes like tyrosine hydroxylase (TH) could result in the synthesis of neurotransmitters such as catecholamines, norepinephrine and epinephrine.

Dorsal Raphe Nuclei (DRN) sections were processed to evaluate the density of 5-HT cells.

After an overnight wash in PBS, sections were incubated with a solution of PBS containing 0.2% Triton X-100 for 30 min and then treated for blocking endogenous peroxidase activity (PBS solution containing methanol/hydrogen peroxide, 1:1, 20 min, at room temperature). After that, nonspecific binding was blocked by normal goat serum (for dopamine) or normal donkey serum (for serotonin) (dilution of 15μl/1ml of PBS; NGS, Vector Laboratories, Burlingame, CA, USA). The sections were then incubated overnight with an α-AADC primary antibody made in rabbit (dilution

1:50000 in a solution of PBS Triton-X100 0.2% pH 7.4 and NGS 2%; Cat. No. TE102, Eugene Tech International, New Jersey, USA) or α-5-HT primary antibody made in goat (dilution 1:8000 in PBS Triton-X100 0.2% pH 7.4 and NGS 2%; Cat. No. 20079, Immunostar, Hudson, USA). After the overnight incubation with the primary antibodies, the sections were incubated for 60' with biotinylated secondary goat anti-rabbit IgG (dilution 1:250 in PBS; Cat. No. BA-1000, Vector Laboratories, Burlingame, USA). After that, the slices were incubated with the avidin-biotin-peroxidase complex (ABC, solution A 1% + solution B 1% in PBS without Na-azide). The peroxidase activity was visualized with a solution containing 0.400 mg/ml of 3,3'-diaminobenzidine (DAB, SIGMA-Aldrich, Milan, Italy) and 0.004% hydrogen peroxide in 0.05 M Tris-HCl buffer pH 7.6. Sections were mounted on chromallum-coated slides, air-dried, cleared in xylene and cover-slipped with Entellan (Merck, Milano, Italy).

6.2.4 Quantitative analysis

All the immunohistochemical experiments included the quantitative analysis of cells: the amount of DA+ cells provided by the α-AADC staining in the VTA and SNpc and 5-HT+ cells in the DRN. Rostro-caudal levels considered for quantitative analysis of DA in the VTA and SNpc were from Bregma -5.20 mm to -5.80 mm and DRN from Bregma -7.30 mm to -8.00 mm (Paxinos and Watson, 1998). For each animal, five (5) levels of VTA/SNpc, and three (3) of DRN were acquired with a NIKON Digital Sight DS-Fi1 video camera connected to a NIKON Eclipse 80i microscope, using a 4x objective. Digital images were processed and analysed automatically by ImageJ (version 1.46r; Wayne Rasband, NIH, Bethesda, MD, USA), using the *Analyze Particles* function of the software. The density of cells at every level was calculated as the number of cells to the area of the appropriate ROI multiplied by 10^4. The mean density of cells at dopamine and serotonin levels was considered for the statistical analysis.

LEVELS OF VTA CONSIDERED

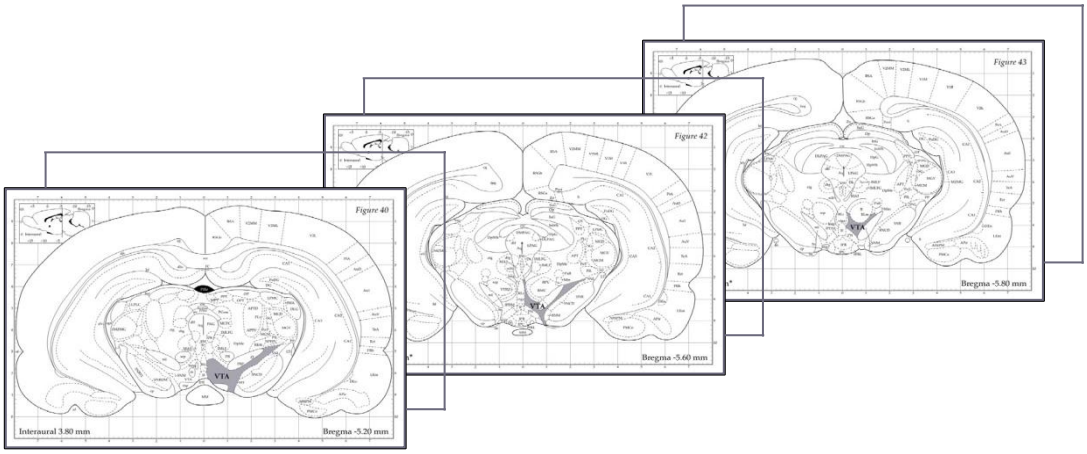


Figure 26: Rostro-caudal levels considered for quantitative analysis of DA in the VTA (from Bregma -5.20 mm to Bregma -5.80 mm; modified from Franklin and Paxinos 1998).

LEVELS OF DRN CONSIDERED

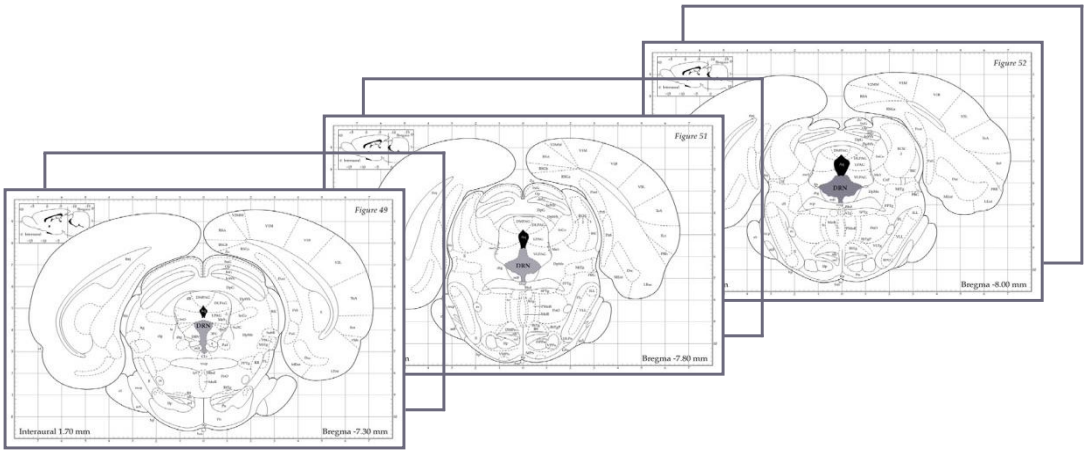


Figure 27: Rostro-caudal levels considered for quantitative analysis of 5-HT in the DRN (from Bregma -7.30 mm to Bregma -8.00 mm; modified from Franklin and Paxinos 1998).

6.2.5 Statistical analysis

The densities of cells obtained from quantitative analysis were processed using the SPSS 24.0 program, with a two-way ANOVA with sex and treatments (CON, DIET, ABA, MSCON, MSDIET, MSABA) as independent variables. For single sex analysis, a three-way ANOVA was used to analyse the effect of stress (MS/NH), activity (ACT/NO ACT) and food intake (FR/NO FR) and their interactions after verifying the normality of the data with the Shapiro-Wilk test. If the value of ANOVA was significant, we evaluated the difference among experimental groups employing the Student Newman-Keuls post hoc test, and the significance was set at $p < 0.05$.

6.3 RESULTS

6.3.1 Density of DA$^+$ cells' in the VTA

The quantitative analysis for the combined group in the VTA showed main effects on the density of DA+ cells given by sex ($F_{(1,78)}$ = 6.78, p = 0.011), treatments ($F_{(5,78)}$ = 3.96, p = 0.003) and by the interaction between the two independent variables ($F_{(5,78)}$ = 3.76, p = 0.004, Table 12).

Among females, a main effect by activity ($F_{(1,45)}$ = 8.908, p < 0.01) and food intake ($F_{(1,45)}$ = 25.185, p < 0.001, Table 13) was observed. The SNK post hoc analysis indicated a significantly increased expression for both ABA groups compared to their respective controls, i.e. FABA > FCON, p < 0.001, and MSFABA > MSFCON, p < 0.001 (figure 28). The results also showed a higher DA+ cells' expression for food restricted maternally separated group, i.e. MSFDIET > MSFCON, p < 0.001 (figure 28).

Although no significant effects were observed among the male groups, there seemed to be a trend of increased expression for the food-restricted and ABA group, especially when maternal separation was absent (figure 28).

Comparing sexes, independently of the maternal separation, a higher expression of DA+ cells was recorded for females to male ABA group, p < 0.001 (figure 28).

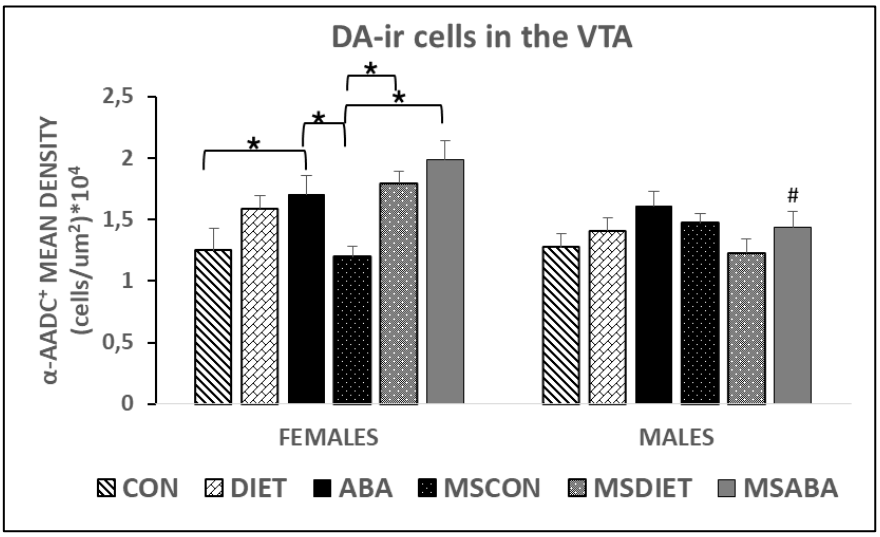


Figure 28: The graph shows the mean density of the expression of DA+ cells in the VTA (expressed in (cells/um2)*10^4). Asterisks (*) indicate significant differences (p < .05) among the differently treated groups (e.g. FCON vs. FABA) and number signs (#) indicate significant sex differences (p < .05) in the same group (e.g. MSFABA vs. MSMABA).
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

6.3.2 Density of DA^+ cells in the SNpc

The quantitative analysis for the combined groups in the SNpc showed main effects on the density of DA+ cells given by sex ($F_{(1,78)}$ = 0.41, p = 0.523), treatments ($F_{(5,78)}$ = 7.86, p < 0.001) and by the interaction between the two independent variables ($F_{(5,78)}$ = 5.30, p < 0.001, Table 12).

In females, comparable to the results obtained in the VTA, food restriction alone or combined with activity increased dopamine cells' expression in the SNpc. Statistically, an increased expression was observed for the maternally separated food-restricted and ABA groups with respect to the control group, i.e. MSFDIET > MSFCON, p < 0.001, and MSFABA > MSFCON, p < 0.001 (figure 29).

In males, a main effect of activity ($F_{(1,37)}$ = 20.194, p < 0.001) and food intake ($F_{(1,37)}$ = 4.805, p < 0.05**,** Table 13**)** was observed following a two-way ANOVA. A significantly increased level of dopamine cells in this region was observed in groups with ABA, independently of neonatal treatment, compared to their respective controls, i.e. MABA > MCON, p < 0.001, and MSMABA > MSMCON, p < 0.001 (figure 29). A greater expression was also shown for the MSMABA compared to only food-restricted group, MSMDIET, p < 0.001 (figure 29). Also, a significant difference in expression levels was observed when MDIET was compared with MSMDIET, p < 0.001 (figure 29).

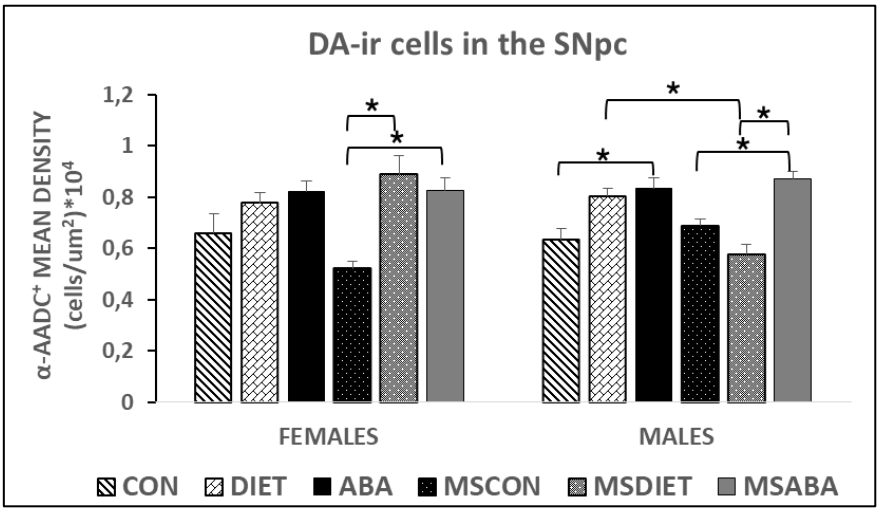


Figure 29: The graph shows the mean density of the expression of DA^+ cells in the SNpc (expressed in (cells/um^2)*10^4). Asterisks (*) indicate significant differences (p < .05) among the differently treated groups (e.g. MSFCON vs. MSFABA). MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

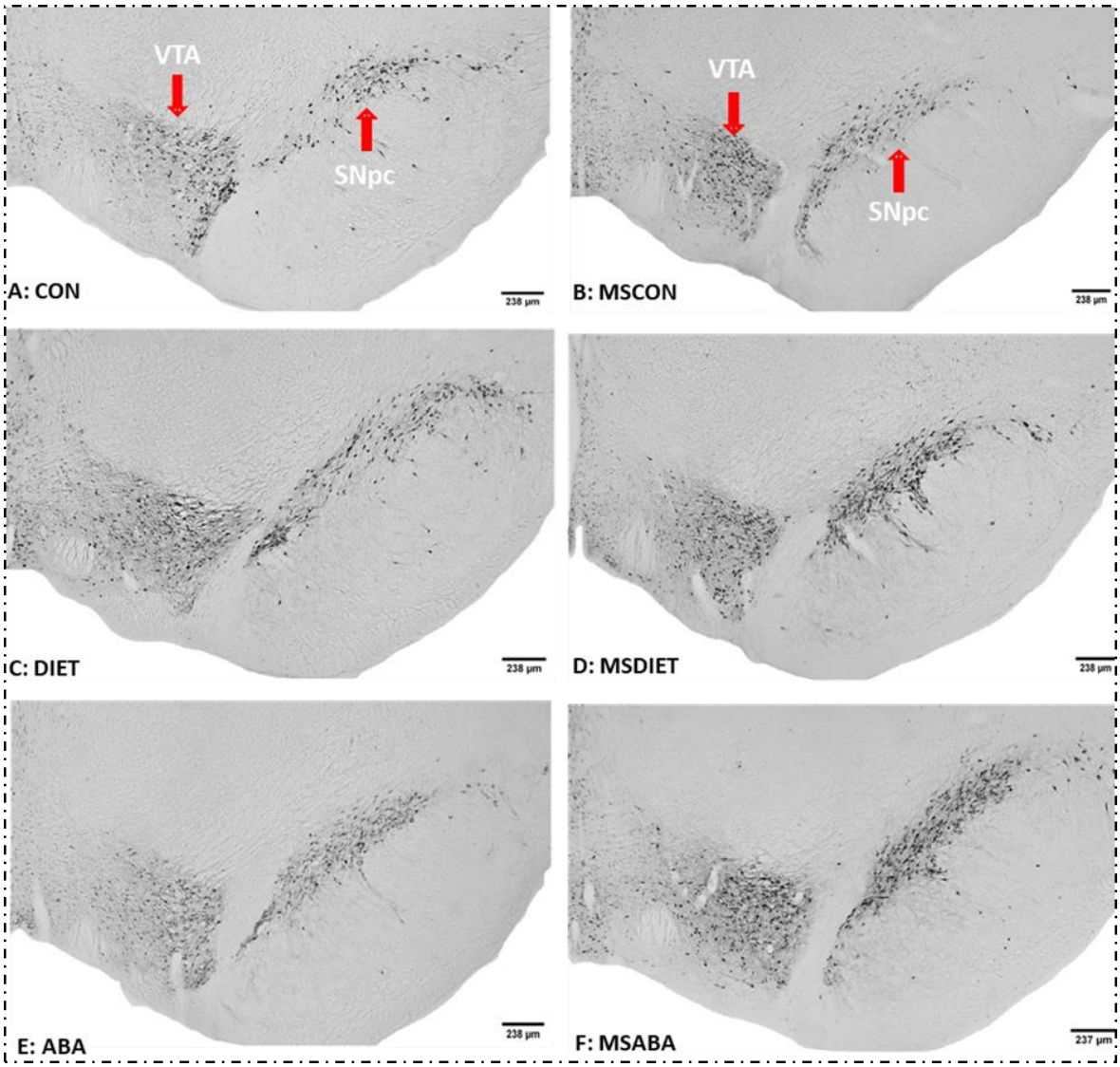


Figure 30: Microphotography illustrating differences in the percentage of DA cells' density in the VTA and SNpc of female groups (Bregma -5.20 mm).
VTA (ventral tegmental area), SNpc (substantia nigra pars compacta)

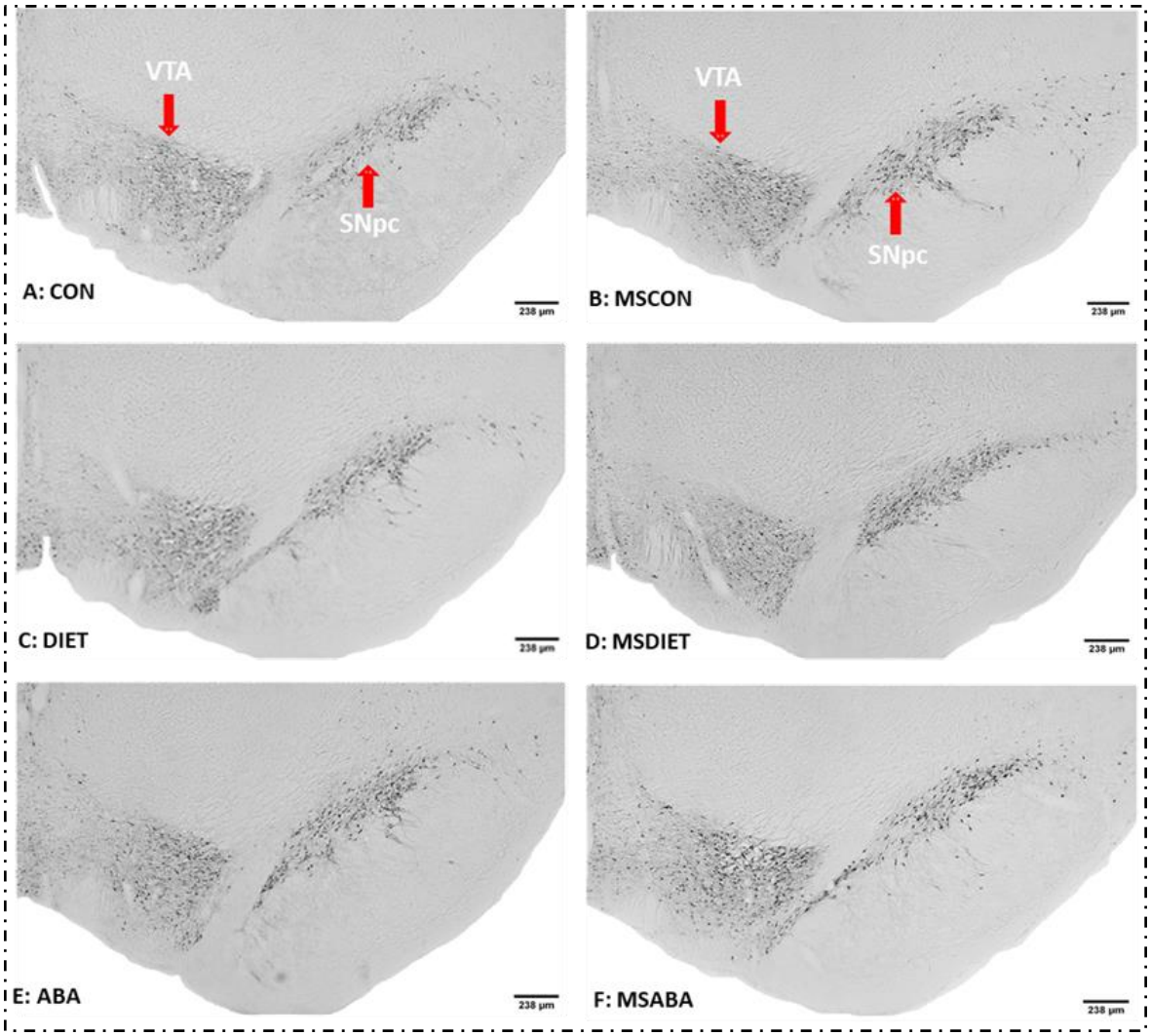


Figure 31: Microphotography illustrating differences in the percentage of DA cells' density in the VTA and SNpc of male groups (Bregma -5.20 mm).
VTA (ventral tegmental area), SNpc (substantia nigra pars compacta)

6.3.3 Density of 5-HT^{+} cells in the DRN

As observed in the VTA and SNpc, there was a main effect of sex ($F_{(1,74)}$ = 13.80, p < 0.001), treatments ($F_{(5,74)}$ = 11.16, p < 0.001) and the interaction between them ($F_{(5,74)}$ = 2.98, p = 0.017) among the experimental groups (Table 12).

Among females, a two-way ANOVA indicated a main effect of stress ($F_{(1,42)}$ = 7.927, p < 0.01), food intake ($F_{(1,42)}$ = 6.716, p < 0.05) and the interactions between these two factors ($F_{(1,42)}$ = 4.914, p < 0.05, Table 13). In addition, a main effect of the interaction between stress and activity was observed ($F_{(1,42)}$ = 6.581, p < 0.05, Table 13). Among non-maternally separated subjects, a significant decline in 5-HT cells' expression was observed in only food-restricted and food restriction coupled with activity groups, i.e. FDIET & FABA < FCON, p = 0.001 (figure 32). Among the maternally separated groups, although the expression was reduced in the DIET group, there was a significant increase in the ABA compared to the DIET group, p = 0.001 (figure 32). More importantly, the expression of the 5-HT cells was higher in the MSFABA than the FABA group, p = 0.001 (figure 32).

A similar effect was observed among the male non-maternally separated groups. A statistically declined level was observed for the only food-restricted and ABA groups compared to the control, p = 0.001 (figure 32), and ABA compared to the only food-restricted group, p = 0.001 (figure 32). Also, the expression of the cells was statistically significant for the MSMABA with respect to the MABA group, p = 0.001, figure 32.

Comparing sexes, independently of the maternal separation and the ABA protocol, 5-HT cells' expression were significantly higher in females compared to males, i.e. FABA > MABA, p = 0.001; MSFABA > MSMABA, p = 0.001 (figure 32).

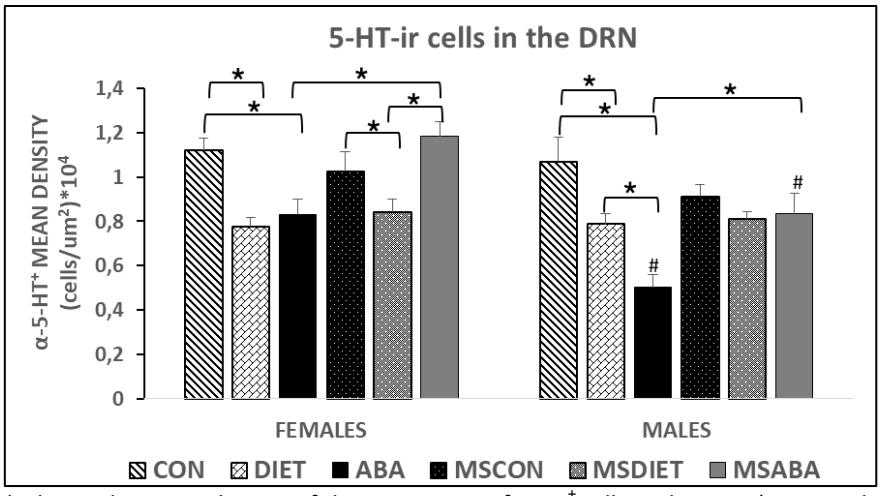


Figure 32: The graph shows the mean density of the expression of 5-HT$^+$ cells in the DRN (expressed in (cells/um^2)*10^4). Asterisks (*) indicate significant differences ($p < .05$) among the differently treated groups (e.g. MSFCON vs. MSFDIET) and number signs (#) indicate significant sex differences ($p < .05$) in the same group (e.g. MSFABA vs. MSMABA).
MS (maternal separation), CON (ad lib. feeding and without wheel access), DIET (food-restricted but without wheel access), ABA (food-restricted with wheel access).

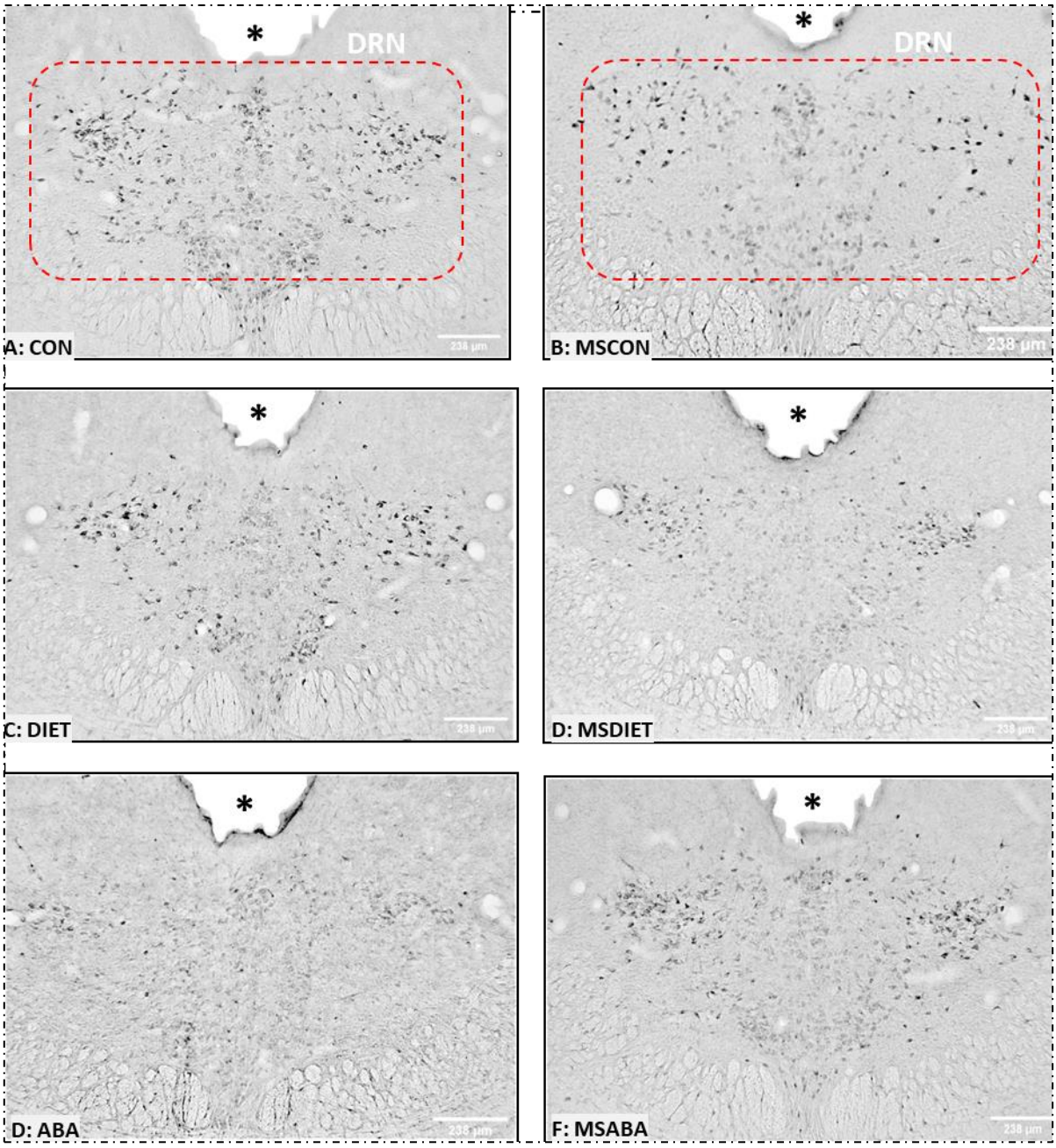


Figure 33: Microphotographs illustrating the differences in the percentage of 5HT cells density in the DRN of female groups (Bregma -7.80 mm).
* (acqueduct)

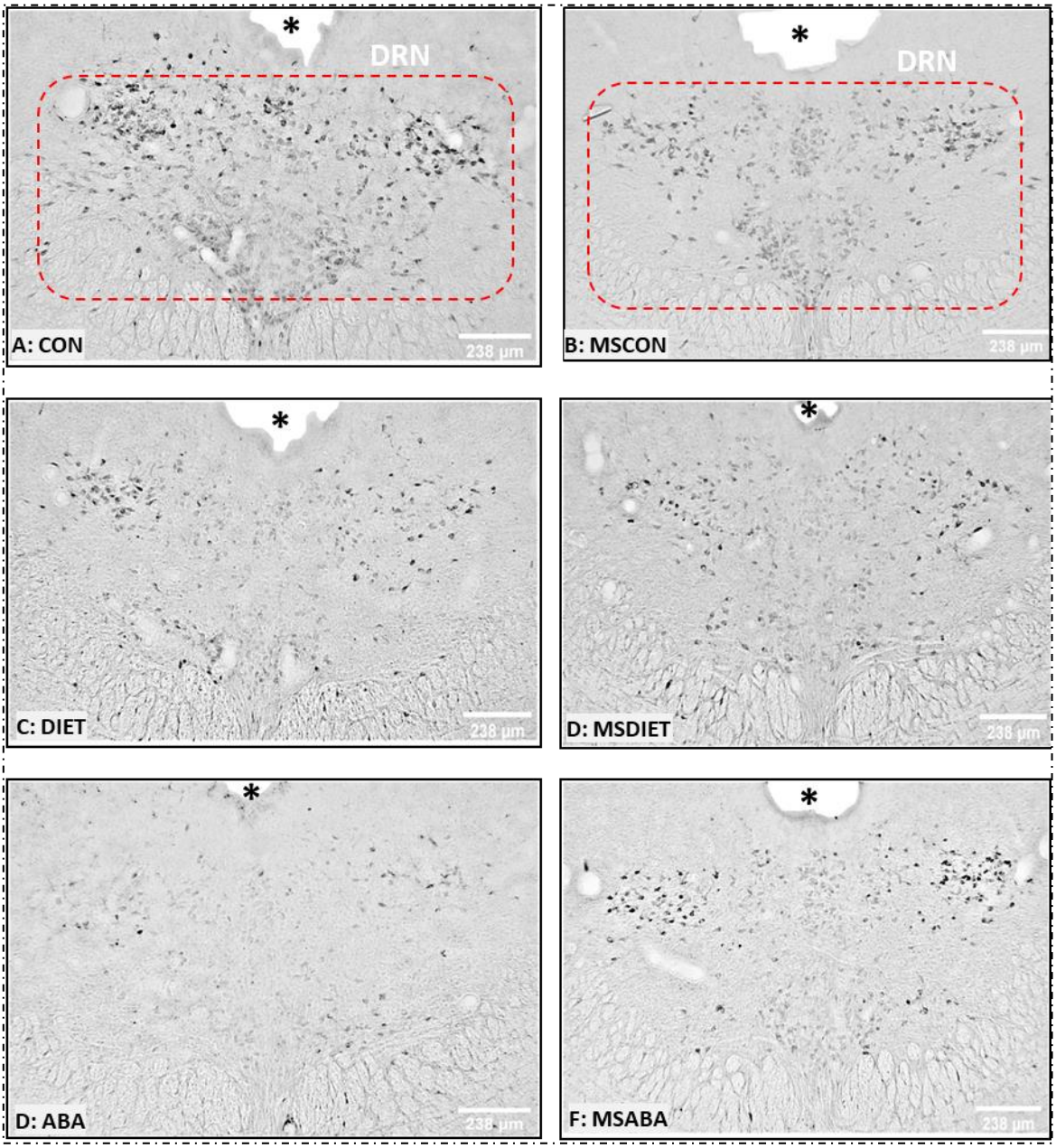


Figure 34: Microphotographs illustrating the differences in the percentage of 5HT cells density in the DRN of male groups (Bregma -7.80 mm).
* (acqueduct)

Table 12. A two-way ANOVA results for the immunohistochemical analysis of the dopaminergic and serotonergic systems.

INDEPENDENT VARIABLES	DOPAMINE VTA	DOPAMINE SNPC	SEROTONIN DRN
SEX	$F_{(1,78)}$=6.78 p = 0.011	$F_{(1,78)}$=0.41 p = 0.523	$F_{(1,74)}$=13.80 p < 0.001
TREATMENT	$F_{(5,78)}$=3.96 p = 0.003	$F_{(5,78)}$=7.86 p < 0.001	$F_{(5,74)}$=11.16 p < 0.001
SEX*TREATMENT	$F_{(5,78)}$=3.76 p = 0.004	$F_{(5,78)}$=5.30 p < 0.001	$F_{(5,74)}$=2.98 p = 0.017

Note: The table shows the results of the ANOVA analysis for the neuroendocrinological systems considered. The independent variables evaluated were the effects of Sex (Female and Male), Treatment (CON, DIET, ABA, MSCON, MSDIET, MSABA), and the interaction between Sex and Treatment. The analysis comprises the immunoreactivity for dopamine in the VTA and SNpc, and serotonin in the DRN. A significant effect is indicated by $p < 0.05$.

Table 13: A three-way ANOVA results for the single-sex immunohistochemical analysis

	FACTORS			INTERACTION	
PARAMETER	**1. STRESS (MS vs NH)**	**2. ACTIVITY (ACT vs NO ACT)**	**3. FOOD INTAKE (FR vs NO FR)**	**1*2**	**1*3**
ALL FEMALES **DOPAMINE**					
Density of DA-ir cells in VTA	n.s	$F_{(1,45)}$ = 8.908, p < 0.01	$F_{(1,45)}$ = 25.185, p < 0.001	n.s	n.s
Density of DA-ir cells in SNpc	n.s	n.s	$F_{(1,45)}$ = 28.308, p < 0.001	n.s	$F_{(1,45)}$ = 5.095, p < 0.05
SEROTONIN					
Density of 5-HT-ir cells in DRN	$F_{(1,42)}$ = 7.927, p < 0.01	n.s	$F_{(1,42)}$ = 6.716, p < 0.05	$F_{(1,42)}$ = 6.581, p < 0.05	$F_{(1,42)}$ = 4.914, p < 0.05
ALL MALES **DOPAMINE**					
Density of DA-ir cells in VTA					
Density of DA-ir cells in SNpc	n.s	$F_{(1,37)}$ = 20.194, p < 0.001	$F_{(1,37)}$ = 4.805, p < 0.05		
SEROTONIN			$F_{(1,36)}$ = 15.146, p < 0.01		
Density of 5-HT-ir cells in DRN	$F_{(1,36)}$ = 5.444, p < 0.05	$F_{(1,36)}$ = 12.012, p < 0.01		$F_{(1,36)}$ = 9.101, p < 0.01	$F_{(1,36)}$ = 6.512, p < 0.05

Note: The table shows the results of the three-way ANOVA for the neuroendocrinological systems considered. The independent variables evaluated were stress (MS/NH), activity (ACT/NO ACT), food intake (FR/NO FR) and the interactions between them. The analysis comprises the immunoreactivity for dopamine in the VTA and SNpc, and serotonin in the DRN. A significant effect is indicated by $p < 0.05$.

6.4 DISCUSSION

The immunohistochemical analysis in the VTA revealed more prominent differences in dopamine neurons expression in females compared to males. This difference was independent of the maternal separation because food restriction alone or ABA induction enhanced expression in both non-handled and maternally separated subjects compared to respective control groups. In the SNpc, a similar incidence was observed in both sexes, especially from the ABA induction. There was a significant increase in DA^+ cells expression in maternally separated ABA compared to control groups and, in males only, between non-handled ABA compared to controls. Interestingly, expression in the SNpc was reduced in MS males when food restricted groups were compared.

Regarding 5-HT, non-handling coupled with DIET or ABA protocol reduced 5-HT expression in both sexes. The neonatal treatment in females and males significantly increased 5-HT immunoreactivity levels compared to non-handled ABA groups. Among ABA groups, independently of maternal separation, 5-HT expression was higher in females than males.

Food shortage or restriction has been observed to elicit stress, affecting the dopaminergic system (Sodersten et al., 2016; Holly et al., 2015). Due to the continued caloric restriction and wheel access availability as in the ABA paradigm (Routtenberg & Kuznesof, 1967), the alteration in the dopaminergic system becomes sustained, reflected in the enhanced levels of DA^+ cells (Aspesi et al., 2021). The analysis in our experiment indicated a higher expression primarily due to the treatment from either food restriction or its combination with activity in both sexes. Thus, we suggest that the significant increase in DA^+ cells underlines the increased activity levels observed on the running wheel. The reduced expression of the DA^+ cells in the groups without ABA, i.e. CON and MSCON, does not support the higher exploratory activity recorded in the behaviour analysis. This discrepancy support the notion that total distance travelled in the classical OF apparatus does not explain exploratory but escape behaviour (Genaro & Schmidek, 2000)

Comparing activity levels in the ABA groups, Chapter 3, paragraph 3.3.3, females were hyperactive compared to males independently of the MS protocol. We observed a significant increase in the expression of DA^+ cells in the VTA for females but not in males. Increased expression in males was observed primarily in the SNpc. The difference in expression levels in these two dopaminergic subpopulations is dependent on the function of these subpopulations (Boekhoudt et al., 2016). The projection of dopamine from the VTA to NAc has been observed to sustain largely locomotor hyperactivity in mice and rats (Boekhoudt et al., 2016), whereas an optogenetic stimulation of SN dopamine neurons in intact mice resulted in subtle movements (Boekhoudt et al., 2016).

Concerning SN dopamine neurons, authors primarily argued in favour of motor coordination but not locomotor hyperactivity (Boekhoudt et al., 2016).

The expression levels of 5HT have been observed to influence dopaminergic activation in an inverse manner (Kapur & Remington, 1996). Correspondingly, in our analysis, the groups with increased dopamine expression had a declined level of 5HT, which was mainly observed in non-handled DIET or ABA of both sexes. The MS procedure, On the other hand, in MS ABA groups, there was a trend of a higher and equal levels of 5HT expression compared to control groups in females and males, respectively. In addition, the MS protocol significantly impacted anxiety by increasing 5HT expression in ABA females and males compared to respective non-handled groups. As reported by Aspesi et al., 2021, MS and ABA protocol synergised to reduce anxiety in females due to enhanced 5HT expression (Aspesi et al., 2021). This influence of the separation protocol could also underline the modulation in general activity levels observed in MS ABA groups. More importantly, the reduced level of tryptophan, the essential amino acid required for 5-HT synthesis, due to the food restriction, could account for the lower levels of 5-HT expression. Incidences of hypophagia and body weight loss in rats, as observed in the ABA group, have been noted in the presence of tryptophan deprivation (Zapata et al., 2018).

The intense activity and reduced food intake which drive the pathological weight loss in AN, is not just a wilful behaviour but, as observed in this part of the experiment, could be underlined by alterations in specific brain regions. The MS protocol provides a protective effect due to its modulation of the alterations observed in these regions when the ABA protocol is induced.

CHAPTER 7: GENERAL CONCLUSIONS

Conclusively, we have demonstrated the interplay between early maternal separation, food restriction and activity to the susceptibility to the ABA protocol. Behavioural alterations and specific brain regions' modulation following the induction of early life stress with and without the ABA protocol have been analysed.

- Briefly, per observation, maternal behaviour towards pups upon reunion appeared inconsistent during the separation days. However, these changes did not significantly affect the subjects' body weight on the weaning day (Chapter 3). Maternal behaviour such as licking, grooming and arched-back nursing increases and decreases during brief handling and MS periods, respectively (Caldji et al., 2000). This condition, in the latter, is observed to increase fearfulness in response to novelty in animal subjects (Caldji et al., 2000). Although the maternal behaviour towards pups normalised during the second week of separation in my experiment, pup-directed behaviours observed in the initial days were reduced.
- Food restriction alone induced weight loss, but pathological weight loss was observed when coupled with activity as in the ABA protocol. Although food restriction plays a significant role in the consequential body weight loss in ABA, as reported in (Chapter 4, paragraph 4.3.2), other authors have observed a reduced decline in the weight loss when animals are only food restricted. In fact, food intake in these subjects normalises and increases with time (Doerries et al., 1991; Carrera et al., 2009; Foldi et al., 2020). For the full expression of the anorexic phenotype, a combination of both food restriction and activity is recommended (Chowdhury et al., 2015; Lamanna et al., 2019).
- Females have been demonstrated to express higher vulnerability to the ABA protocol than males, as confirmed by the significantly higher levels of running activity (Chapter 4, paragraph 4.3.3). Maternal separation conferred a protective effect, especially on activity levels during the ABA protocol, evidenced by the increasing trend of activity and time spent on the wheel in male and female non-handled compared to the MS groups. In a recent published work from Farinetti et al., 2020, although ABA in the presence of MS resulted in an increase in activity levels there was a significant reduction in anxiety parameters in females (Farinetti et al., 2020). Similarly, Carrera and colleagues have reported a protective effect of the MS protocol in ABA females although no effect was observed in males (Carrera et al., 2009).
- The synergy between different activity periods underlined the observed hyperactivity in a sex-dependent manner: FAA and NA for females (most active group) and FAA and PPA for

the hyperactive male group. From the literature, activity during the FAA period has been observed to spike up exponentially in the ABA protocol (Hillebrand et al., 2005; Wu et al., 2014; Klenotich et al., 2012), and attempt to reduce this activity period has resulted in reducing the susceptibility to the ABA protocol. Wu et al. have also commented on the effect of the PPA in sustaining the intense activity recorded during the ABA procedure (Wu et al., 2014).

- Behaviour analysis using the OF and EPM indicated reduced anxiety following food restriction or ABA treatment. Separation during the early postnatal days reduced anxiogenic parameters in most subjects. This resonates with the report of Farinetti et al., 2020, since MS + ABA resulted in an anxiolytic effect in females. Although authors observed increased anxiety parameters in MS + ABA males, the male ABA group compared to controls were also anxiolytic (Farinetti et al., 2020).
- Immunohistochemically, the observed difference in the expression levels of DA and 5HT cells was supported by the separate and combined effects of the MS and ABA protocol. Significantly increased levels of DA^+ cells were observed in groups that were food restricted only or coupled with activity. Aside from the inherent trait of increased activity levels observed in female rats (Rosenfeld, 2017), the higher expression levels of DA, mainly in the VTA, of female ABA groups could underline the differences in hyperactivity between the two sexes. Dopamine expression, mainly in the VTA, is implicated in pronounced and long-lasting hyperactive phenotype (Boekhoudt et al., 2016).
- The MS stress coupled with food restriction or ABA generally reduced the expression of 5-HT^+ cells in the DRN. Interestingly, 5HT expression was significantly higher in both male and female ABA groups that were maternally separated than subjects without maternal separation.

Taken together, this thesis has confirmed the validity of the ABA protocol in the analysis of human AN due to the observed core features of the disorder. Although the early adverse environment has deleterious effects in most cases, it could also impact positively. Behavioural changes observed in AN individuals is underlined by alterations in specific brain regions.

Although there is substantial literature on the effect of adverse early environment on the susceptibility of AN in human and animal studies (Carrera et al., 2006 & 2009: Hancock et al., 2009, Farinetti et al., 2020), not much work has been focused on analysing the impact of the different

activity periods in the ABA protocol in the presence of early maternal separation. I want to highlight that this work, if not the first, is among the first to analyse the impact of activity in different periods combining early life stress with ABA protocol in both sexes.

Different systems work in tandem to manifest alterations observed in several human disorders. Although both systems investigated in this thesis are instrumental in the pathophysiology and behavioural dysregulations of AN both in humans and animals, it would be worthwhile in future studies with the ABA model to include other circuits such as the hypothalamic system, which is a crucial player in the regulation of appetite/food intake. Further studies in analysing peripheral signals involved in feeding behaviour and energy homeostasis such as leptin and ghrelin would throw more light on the underlying effects observed.

CHAPTER 8: REFERENCES

1. Abbate-Daga, G., Amianto, F., Delsedime, N., De-Bacco, C., & Fassino, S. (2013). Resistance to treatment and change in anorexia nervosa [corrected]: a clinical overview. *BMC Psychiatry*, *13*, 294. https://doi.org/10.1186/1471-244X-13-294
2. Aisa, B., Tordera, R., Lasheras, B., del Río, J., & Ramírez, M. J. (2007). Cognitive impairment associated to HPA axis hyperactivity after maternal separation in rats. *Psychoneuroendocrinology*, *32*(3), 256–266. https://doi.org/10.1016/j.psyneuen.2006.12.013
3. Allen, P. J., Jimerson, D. C., Kanarek, R. B., & Kocsis, B. (2017). Impaired reversal learning in an animal model of anorexia nervosa. *Physiology and Behavior*, *179*, 313–318. https://doi.org/10.1016/j.physbeh.2017.06.013
4. Alliot, J., Boghossian, S., Jourdan, D., Veyrat-Durebex, C., Pickering, G., Meynial-Denis, D., & Gaumet, N. (2002). The LOU/c/jall Rat as an Animal Model of Healthy Aging? In *Journal of Gerontology* (Vol. 57, Issue 8). https://academic.oup.com/biomedgerontology/article-abstract/57/8/B312/556752
5. Amianto, F., Northoff, G., Abbate Daga, G., Fassino, S., & Tasca, G. A. (2016). Is Anorexia Nervosa a Disorder of the Self? A Psychological Approach. *Frontiers in Psychology*, *7*, 849. https://doi.org/10.3389/fpsyg.2016.00849
6. Amianto, F., Siccardi, S., Abbate-Daga, G., Marech, L., Barosio, M., & Fassino, S. (2012). Does anger mediate between personality and eating symptoms in bulimia nervosa? *Psychiatry Research*, *200*(2–3), 502–512. https://doi.org/10.1016/j.psychres.2012.07.036
7. Aspesi, D., Farinetti, A., Marraudino, M., Morgan, G. S. K., Marzola, E., Abbate-Daga, G., & Gotti, S. (2021). Maternal separation alters the reward system of activity-based anorexia rats. Psychoneuroendocrinology, 133. https://doi.org/10.1016/j.psyneuen.2021.105393
8. Bailoo, J. D., Voelkl, B., Varholick, J., Novak, J., Murphy, E., Rosso, M., Palme, R., & Würbel, H. (2020). Effects of weaning age and housing conditions on phenotypic differences in mice. *Scientific Reports*, *10*(1). https://doi.org/10.1038/s41598-020-68549-3
9. Bélanger, M., Allaman, I., & Magistretti, P. J. (2011). Brain energy metabolism: Focus on Astrocyte-neuron metabolic cooperation. In Cell Metabolism (Vol. 14, Issue 6, pp. 724–738). https://doi.org/10.1016/j.cmet.2011.08.016
10. Belmonte, L., Achamrah, N., Nobis, S., Guérin, C., Riou, G., Bôle-Feysot, C., Boyer, O., Richard, V., do Rego, J. C., Déchelotte, P., Goichon, A., & Coëffier, M. (2016). A role for intestinal TLR4-driven inflammatory response during activity-based anorexia. *Scientific Reports*, *6*. https://doi.org/10.1038/srep35813
11. Berner, L. A., Brown, T. A., Lavender, J. M., Lopez, E., Wierenga, C. E., & Kaye, W. H. (2019). Neuroendocrinology of reward in anorexia nervosa and bulimia nervosa: Beyond leptin and ghrelin. In *Molecular and Cellular Endocrinology* (Vol. 497). Elsevier Ireland Ltd. https://doi.org/10.1016/j.mce.2018.10.018
12. Biagini G, Pich EM, Carani C, Marrama P, Agnati LF. (1998). Postnatal maternal separation during the stress hyporesponsive period enhances the adrenocortical response to novelty in adult rats by affecting feedback regulation in the CA1 hippocampal field. Int J Dev Neurosci. (3-4) 187-97. doi: 10.1016/s0736-5748(98)00019-7. PMID: 9785115.
13. Blundell, J. E. (1984). *SEROTONIN AND APPETITE* (Vol. 23, Issue 12B).

14. Blythe, S. N., Atherton, J. F., & Bevan, M. D. (2007). Synaptic activation of dendritic AMPA and NMDA receptors generates transient high-frequency firing in substantia nigra dopamine neurons in vitro. *Journal of Neurophysiology*, *97*(4), 2837–2850. https://doi.org/10.1152/jn.01157.2006
15. Boakes, R. A., Dwyer, D., Juraskova, I., Mills, K., Whitford, T., Baysari, M., Hughes, S., Dennison, M., & Fardell, J. (2007). Self-Starvation in the Rat: Running versus Eating. *The Spanish Journal of Psychology Copyright*, *10*(2), 251–257.
16. Boekhoudt, L., Omrani, A., Luijendijk, M. C. M., Wolterink-Donselaar, I. G., Wijbrans, E. C., van der Plasse, G., & Adan, R. A. H. (2016). Chemogenetic activation of dopamine neurons in the ventral tegmental area, but not substantia nigra, induces hyperactivity in rats. European Neuropsychopharmacology, 26(11), 1784–1793. https://doi.org/10.1016/j.euroneuro.2016.09.003
17. Boersma, G. J., Treesukosol, Y., Cordner, Z. A., Kastelein, A., Choi, P., Moran, T. H., & Tamashiro, K. L. (2016). Exposure to activity-based anorexia impairs contextual learning in weight-restored rats without affecting spatial learning, taste, anxiety, or dietary-fat preference. *The International journal of eating disorders*, *49*(2), 167–179. https://doi.org/10.1002/eat.22489
18. Bulik, C., Yilmaz, Z., & Hardaway, A. (2015). Genetics and epigenetics of eating disorders. *Advances in Genomics and Genetics*, 131. https://doi.org/10.2147/agg.s55776
19. Caldji, C., Francis, D., Sharma, S., Plotsky, P. M., & Meaney, M. J. (2000). The Effects of Early Rearing Environment on the Development of GABA A and Central Benzodiazepine Receptor Levels and Novelty-Induced Fearfulness in the Rat. In *Neuropsychopharmacology* (Vol. 22, Issue 3).
20. Caldji, C., Tannenbaum, B., Sharma, S., Francis, D., Plotsky, P. M., & Meaney, M. J. (1998). Maternal care during infancy regulates the development of neural systems mediating the expression of fearfulness in the rat. In Neurobiology (Vol. 95). www.pnas.org.
21. Carr, K. D., Tsimberg, Y., Berman, Y., & Yamamoto, N. (2003). Evidence of increased dopamine receptor signaling in food-restricted rats. *Neuroscience*, *119*(4), 1157–1167. https://doi.org/10.1016/S0306-4522(03)00227-6
22. Carrasco, G. A., & van de Kar, L. D. (2003). Neuroendocrine pharmacology of stress. *European Journal of Pharmacology*, *463*(1–3), 235–272. https://doi.org/10.1016/S0014-2999(03)01285-8
23. Carrera, O., Cerrato, M., Sanchez, A., & Gutierrez, E. (2009). Long maternal separation has protective effects in rats exposed to activity-based anorexia. Developmental Psychobiology, 51(8), 616–624. https://doi.org/10.1002/dev.20396
24. Carrera, O., Gutiérrez, E., & Boakes, R. A. (2006). Early handling reduces vulnerability of rats to activity-based anorexia. Developmental Psychobiology, 48(7), 520–527. https://doi.org/10.1002/dev.20175
25. Carta, M., Fadda, F., & Stancampiano, R. (2006). Tryptophan-deficient diet increases the neurochemical and behavioral response to amphetamine. *Brain Research*, *1094*(1), 86–91. https://doi.org/10.1016/j.brainres.2006.03.118
26. Carter, J. C., Blackmore, E., Sutandar-Pinnock, K., & Woodside, D. B. (2004). Relapse in anorexia nervosa: A survival analysis. *Psychological Medicine*, *34*(4), 671–679. https://doi.org/10.1017/S0033291703001168

27. Caslini, M., Bartoli, F., Crocamo, C., Dakanalis, A., Clerici, M., & Carrà, G. (2016). Disentangling the association between child abuse and eating disorders: A systematic review and meta-analysis. In *Psychosomatic Medicine* (Vol. 78, Issue 1, pp. 79–90). Lippincott Williams and Wilkins. https://doi.org/10.1097/PSY.0000000000000233
28. Casper, R. C., Sullivan, E. L., & Tecott, L. (2008). Relevance of animal models to human eating disorders and obesity. In *Psychopharmacology* (Vol. 199, Issue 3, pp. 313–329). https://doi.org/10.1007/s00213-008-1102-2
29. Chagra, S. L., Zavala, J. K., Hall, M. v., & Gosselink, K. L. (2011). Acute and repeated restraint differentially activate orexigenic pathways in the rat hypothalamus. *Regulatory Peptides*, *167*(1), 70–78. https://doi.org/10.1016/j.regpep.2010.11.006
30. Chowdhury, T. G., Chen, Y. W., & Aoki, C. (2015). Using the activity-based anorexia rodent model to study the neurobiological basis of anorexia nervosa. Journal of Visualized Experiments, 2015(104). https://doi.org/10.3791/52927
31. Connan, F., Murphy, F., Connor, S. E. J., Rich, P., Murphy, T., Bara-Carill, N., Landau, S., Krljes, S., Ng, V., Williams, S., Morris, R. G., Campbell, I. C., & Treasure, J. (2006). Hippocampal volume and cognitive function in anorexia nervosa. *Psychiatry Research - Neuroimaging*, *146*(2), 117–125. https://doi.org/10.1016/j.pscychresns.2005.10.006
32. Corwin, R. L., & Buda-Levin, A. (2004). Behavioral models of binge-type eating. *Physiology and Behavior*, *82*(1), 123–130. https://doi.org/10.1016/j.physbeh.2004.04.036
33. Doerries, L. E., Stanley, E. Z., Aravicht~, P. F., Doerries, L. E., Stanley, E. Z., & Aravich, P. F. (1991). Activity-Based Anorexia: Relationship to Gender and Activity-Stress Ulcers. In Physiology & Behavior (Vol. 50).
34. Duncan, L., Yilmaz, Z., Gaspar, H., Walters, R., Goldstein, J., Anttila, V., Bulik-Sullivan, B., Ripke, S., Thornton, L., Hinney, A., Daly, M., Sullivan, P. F., Zeggini, E., Breen, G., Bulik, C. M., Duncan, L., Yilmaz, Z., Gaspar, H., Walters, R., … Bulik, C. M. (2017). Significant locus and metabolic genetic correlations revealed in genome-wide association study of anorexia nervosa. *American Journal of Psychiatry*, *174*(9), 850–858. https://doi.org/10.1176/appi.ajp.2017.16121402
35. Fairburn, C. G., & Patel, V. (2014). The global dissemination of psychological treatments: A road map for research and practice. *The American Journal of Psychiatry, 171*(5), 495–498. https://doi.org/10.1176/appi.ajp.2013.13111546
36. Farinetti, A., Aspesi, D., Marraudino, M., Marzola, E., Amianto, F., Abbate-Daga, G., & Gotti, S. (2020). Sexually dimorphic behavioral effects of maternal separation in anorexic rats. Developmental Psychobiology, 62(3), 297–309. https://doi.org/10.1002/dev.21909
37. Ferrario, C. R., Labouèbe, G., Liu, S., Nieh, E. H., Routh, V. H., Xu, S., & O'Connor, E. C. (2016). Homeostasis meets motivation in the battle to control food intake. *Journal of Neuroscience*, *36*(45), 11469–11481. https://doi.org/10.1523/JNEUROSCI.2338-16.2016
38. Ferreira, A., Agrati, D., Uriarte, N., Pereira, M., Cruz-Morales, S. E., Pedro Arriaga-Ramírez, J. C., & Zuluaga, M. J. (2012). *The rat as a model for studying maternal behavior Perinatal Pediatrics View project The rat as a model for studying maternal behavior*. https://www.researchgate.net/publication/285746321

39. Foldi, C. J., Liknaitzky, P., Williams, M., & Oldfield, B. J. (2020). Rethinking Therapeutic Strategies for Anorexia Nervosa: Insights From Psychedelic Medicine and Animal Models. In Frontiers in Neuroscience (Vol. 14). Frontiers Media S.A. https://doi.org/10.3389/fnins.2020.00043
40. Foldi, C. J., Milton, L. K., & Oldfield, B. J. (2017). The Role of Mesolimbic Reward Neurocircuitry in Prevention and Rescue of the Activity-Based Anorexia (ABA) Phenotype in Rats. *Neuropsychopharmacology*, *42*(12), 2292–2300. https://doi.org/10.1038/npp.2017.63
41. Frank, G. K. W., Collier, S., Shott, M. E., & O'Reilly, R. C. (2016). Prediction error and somatosensory insula activation in women recovered from anorexia nervosa. *Journal of Psychiatry and Neuroscience*, *41*(5), 304–311. https://doi.org/10.1503/jpn.150103
42. Frank, G. K., Bailer, U. F., Henry, S. E., Drevets, W., Meltzer, C. C., Price, J. C., Mathis, C. A., Wagner, A., Hoge, J., Ziolko, S., Barbarich-Marsteller, N., Weissfeld, L., & Kaye, W. H. (2005). Increased dopamine D2/D3 receptor binding after recovery from anorexia nervosa measured by positron emission tomography and [11C]raclopride. *Biological Psychiatry*, *58*(11), 908–912. https://doi.org/10.1016/j.biopsych.2005.05.003
43. Franko, D. L., Keshaviah, A., Eddy, K. T., Krishna, M., Davis, M. C., Keel, P. K., & Herzog, D. B. (2013). A longitudinal investigation of mortality in anorexia nervosa and bulimia nervosa. *American Journal of Psychiatry*, *170*(8), 917–925. https://doi.org/10.1176/appi.ajp.2013.12070868
44. Frintrop, L., Liesbrock, J., Paulukat, L., Johann, S., Kas, M. J., Tolba, R., Heussen, N., Neulen, J., Konrad, K., Herpertz-Dahlmann, B., Beyer, C., & Seitz, J. (2018). Reduced astrocyte density underlying brain volume reduction inactivity-based anorexia rats. *World Journal of Biological Psychiatry*, *19*(3), 225–235. https://doi.org/10.1080/15622975.2016.1273552
45. Fujimoto, T., Kubo, K., Nishikawa, Y., & Aou, S. (2014). Brief neonatal handling alters sexually dimorphic behaviors in adult rats. Journal of Integrative Neuroscience, 13(1), 61–70. https://doi.org/10.1142/S0219635214500046
46. Genaro, G., & Schmidek, W.R. (2000). Exploratory Activity of Rats in Three Different Environments. Ethology, 106, 849-859.
47. Gonzalez, L. E., & File, S. E. (1997). A Five Minute Experience in the Elevated Plus-Maze Alters the State of the Benzodiazepine Receptor in the Dorsal Raphe Nucleus.
48. Grzelak, T., Dutkiewicz, A., Paszynska, E., Dmitrzak-Weglarz, M., Slopien, A., & Tyszkiewicz-Nwafor, M. (2017). Neurobiochemical and psychological factors influencing the eating behaviors and attitudes in anorexia nervosa. In *Journal of Physiology and Biochemistry* (Vol. 73, Issue 2, pp. 297–305). Springer Netherlands. https://doi.org/10.1007/s13105-016-0540-2
49. Gutiérrez, E., Vázquez, R. & Boakes, R.A. (2002). Activity-based anorexia: Ambient temperature has been a neglected factor. *Psychonomic Bulletin & Review* 9, 239–249. https://doi.org/10.3758/BF03196278
50. Haleem, D. J. (2017). Improving therapeutics in anorexia nervosa with tryptophan. In *Life Sciences* (Vol. 178, pp. 87–93). Elsevier Inc. https://doi.org/10.1016/j.lfs.2017.04.015
51. Hancock, S. D., & Grant, V. L. (2009). Sexually dimorphic effects of postnatal treatment on the development of activity-based anorexia in adolescent and adult rats. Developmental Psychobiology, 51(8), 679–695. https://doi.org/10.1002/dev.20403

52. Hao, S., Avraham, Y., Bonne, O., & Berry, E. M. (n.d.). *Separation-induced body weight loss, impairment in alternation behavior, and autonomic tone: effects of tyrosine*. www.elsevier.com/locate/pharmbiochembeh
53. Harrington, B. C., Jimerson, M., & Jimerson, D. C. (2015). Initial Evaluation, Diagnosis, and Treatment of Anorexia Nervosa and Bulim<FEFF>ia Nervosa. In *American Family Physician www.aafp.org/afp* (Vol. 91, Issue 1). www.aafp.org/afp.
54. Hebebrand, J., Exner, C., Hebebrand, K., Holtkamp, C., Casper, R. C., Remschmidt, H., Herpertz-Dahlmann, B., & Klingenspor, M. (2003). Hyperactivity in patients with anorexia nervosa and in semistarved rats: Evidence for a pivotal role of hypoleptinemia. *Physiology and Behavior*, *79*(1), 25–37. https://doi.org/10.1016/S0031-9384(03)00102-1
55. Heim, C., & Nemeroff, C. B. (2001). The Role of Childhood Trauma in the Neurobiology of Mood and Anxiety Disorders: Preclinical and Clinical Studies. In *Biol Psychiatry* (Vol. 49).
56. Hilbert, A., Vögele, C., Tuschen-Caffier, B., & Hartmann, A. S. (2011). Psychophysiological responses to idiosyncratic stress in bulimia nervosa and binge eating disorder. *Physiology & behavior*, *104*(5), 770–777. https://doi.org/10.1016/j.physbeh.2011.07.013
57. Hillebrand, J. J. G., van Elburg, A. A., Kas, M. J. H., van Engeland, H., & Adan, R. A. H. (2005). Olanzapine Reduces Physical Activity in Rats Exposed to Activity-Based Anorexia: Possible Implications for Treatment of Anorexia Nervosa? https://doi.org/10.1016/j.biosych.2005.04.008
58. Holly, E. N., Debold, J. F., & Miczek, K. A. (2015). Increased mesocorticolimbic dopamine during acute and repeated social defeat stress: Modulation by corticotropin releasing factor receptors in the ventral tegmental area. *Psychopharmacology*, *232*(24), 4469–4479. https://doi.org/10.1007/s00213-015-4082-z
59. Hu, H. (2016). Reward and Aversion. *Annual Review of Neuroscience*, *39*, 297–324. https://doi.org/10.1146/annurev-neuro-070815-014106
60. Jaimes-Hoy, L., Romero, F., Charli, J. L., & Joseph-Bravo, P. (2019). Sex dimorphic responses of the hypothalamus–pituitary–thyroid axis to maternal separation and palatable diet. *Frontiers in Endocrinology*, *10*(JULY). https://doi.org/10.3389/fendo.2019.00445
61. Jans, J. E., & Woodside, B. C. (1990). *Nest Temperature: Effects on Maternal Behavior, Pup Development, and Interactions with Handling*. Developmental Psychobiology 23(6):519-534
62. Kalinichev, M., Easterling, K. W., Plotsky, P. M., & Holtzman, S. G. (2002). Long-lasting changes in stress-induced corticosterone response and anxiety-like behaviors as a consequence of neonatal maternal separation in Long-Evans rats. www.elsevier.com/locate/pharmbiochembeh
63. Kapur, S., & Remington, G. (1996). Serotonin-dopamine interaction and its relevance to schizophrenia. The American Journal of Psychiatry, 153(4), 466–476. https://doi.org/10.1176/ajp.153.4.466
64. Kaye, W. H., Frank, G. K. W., & Mcconaha, C. (1999). *Altered Dopamine Activity after Recovery from Restricting-Type Anorexia Nervosa* (Vol. 21, Issue 4).
65. Kaye, W. H., Frank, G. K., Bailer, U. F., Henry, S. E., Meltzer, C. C., Price, J. C., Mathis, C. A., & Wagner, A. (2005). Serotonin alterations in anorexia and bulimia nervosa: New insights from imaging studies. *Physiology and Behavior*, *85*(1), 73–81. https://doi.org/10.1016/j.physbeh.2005.04.013

66. Kelley, A. E., Baldo, B. A., Pratt, W. E., & Will, M. J. (2005). Corticostriatal-hypothalamic circuitry and food motivation: Integration of energy, action and reward. *Physiology and Behavior*, *86*(5), 773–795. https://doi.org/10.1016/j.physbeh.2005.08.066
67. Klenotich, S. J., Seiglie, M. P., McMurray, M. S., Roitman, J. D., le Grange, D., Dugad, P., & Dulawa, S. C. (2012). Olanzapine, but not fluoxetine, treatment increases survival in activity-based anorexia in mice. Neuropsychopharmacology, 37(7), 1620–1631. https://doi.org/10.1038/npp.2012.7
68. Knoll, S., Föcker, M., & Hebebrand, J. (2014). Essstörungen [Changes to the classification of Eating Disorders in DSM-5]. *Zeitschrift fur Kinder- und Jugendpsychiatrie und Psychotherapie*, *42*(5), 361–368. https://doi.org/10.1024/1422-4917/a000311
69. Lamanna, J., Sulpizio, S., Ferro, M., Martoni, R., Abutalebi, J., & Malgaroli, A. (2019). Behavioral assessment of activity-based-anorexia: how cognition can become the drive wheel. In Physiology and Behavior (Vol. 202, pp. 1–7). Elsevier Inc. https://doi.org/10.1016/j.physbeh.2019.01.016
70. Lammel, S., Steinberg, E. E., Földy, C., Wall, N. R., Beier, K., Luo, L., & Malenka, R. C. (2015). Diversity of transgenic mouse models for selective targeting of midbrain dopamine neurons. *Neuron*, *85*(2), 429–438. https://doi.org/10.1016/j.neuron.2014.12.036
71. Lesch, K. P., Merschdorf, U., & Lesch, K. P. (2000). Impulsivity, Aggression, and Serotonin: A Molecular Psychobiological Perspective. In *Behavioral Sciences and the Law Behav. Sci. Law* (Vol. 18).
72. Leventopoulos, M., Rüedi-Bettschen, D., Knuesel, I., Feldon, J., Pryce, C. R., & Opacka-Juffry, J. (2007). Long-term effects of early life deprivation on brain glia in Fischer rats. *Brain Research*, *1142*(1), 119–126. https://doi.org/10.1016/j.brainres.2007.01.039
73. Lindfors, C., Nilsson, I. A. K., Garcia-Roves, P. M., Zuberi, A. R., Karimi, M., Donahue, L. R., Roopenian, D. C., Mulder, J., Uhlén, M., Ekström, T. J., Davisson, M. T., Hökfelt, T. G. M., Schalling, M., & Johansen, J. E. (2011). Hypothalamic mitochondrial dysfunction associated with anorexia in the anx/anx mouse. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(44), 18108–18113. https://doi.org/10.1073/pnas.1114863108
74. Madra, M., & Zeltser, L. M. (2016). BDNF-Val66Met variant and adolescent stress interact to promote susceptibility to anorexic behavior in mice. *Translational Psychiatry*, *6*. https://doi.org/10.1038/tp.2016.35
75. Mällo, T., Alttoa, A., Kõiv, K., Tõnissaar, M., Eller, M., & Harro, J. (2007). Rats with persistently low or high exploratory activity: Behaviour in tests of anxiety and depression, and extracellular levels of dopamine. Behavioural Brain Research, 177(2), 269–281. https://doi.org/10.1016/j.bbr.2006.11.022
76. Maltais, L. J., Lane, P. W., & Beamer, W. G. (1984). Anorexia, a recessive mutation causing starvation in preweanling mice. In *The Journal of Heredity* (Vol. 75).
77. Mann J. J. (1999). Role of the serotonergic system in the pathogenesis of major depression and suicidal behavior. *Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology*, *21*(2 Suppl), 99S–105S. https://doi.org/10.1016/S0893-133X(99)00040-8
78. Marzilli, E., Cerniglia, L., & Cimino, S. (2018). A narrative review of binge eating disorder in adolescence: prevalence, impact, and psychological treatment strategies. *Adolescent Health, Medicine and Therapeutics*, *Volume 9*, 17–30. https://doi.org/10.2147/ahmt.s148050

79. Mela, V., Llorente-Berzal, Á., Díaz, F., Argente, J., Viveros, M. P., & Chowen, J. A. (2012). Maternal Deprivation Exacerbates the Response to a High Fat Diet in a Sexually Dimorphic Manner. *PLoS ONE*, *7*(11). https://doi.org/10.1371/journal.pone.0048915
80. Mistlberger, R. E., & Mumby, D. G. (1992). The limbic system and food-anticipatory circadian rhythms in the rat" ablation and dopamine blocking studies. In *Behavioural Brahl Research* (Vol. 47).
81. Nicholls, W., Devonport, T. J., & Blake, M. (2016). The association between emotions and eating behaviour in an obese population with binge eating disorder. *Obesity reviews : an official journal of the International Association for the Study of Obesity*, *17*(1), 30–42. https://doi.org/10.1111/obr.12329
82. Nilsson, I. A. K., Lindfors, C., Schalling, M., Hökfelt, T., & Johansen, J. E. (2013). Anorexia and Hypothalamic Degeneration. In *Vitamins and Hormones* (Vol. 92, pp. 27–60). Academic Press Inc. https://doi.org/10.1016/B978-0-12-410473-0.00002-7
83. Nishi, M., Horii-Hayashi, N., & Sasagawa, T. (2014). Effects of early life adverse experiences on the brain: Implications from maternal separation models in rodents. In *Frontiers in Neuroscience* (Issue 8 JUN). Frontiers Research Foundation. https://doi.org/10.3389/fnins.2014.00166
84. Novelle, M. G., & Diéguez, C. (2018). Food addiction and binge eating: Lessons learned from animal models. In *Nutrients* (Vol. 10, Issue 1). MDPI AG. https://doi.org/10.3390/nu10010071
85. Nunn, K., Frampton, I. J., Gordon, I., & Lask, B. (2008). The fault is not in her parents but in her insula - A neurobiological hypothesis of anorexia nervosa. *European Eating Disorders Review*, *16*(5), 355–360. https://doi.org/10.1002/erv.890
86. O'Hara, C. B., Campbell, I. C., & Schmidt, U. (2015). A reward-centred model of anorexia nervosa: A focussed narrative review of the neurological and psychophysiological literature. In *Neuroscience and Biobehavioral Reviews* (Vol. 52, pp. 131–152). Elsevier Ltd. https://doi.org/10.1016/j.neubiorev.2015.02.012
87. Oberndorfer, T., Simmons, A., McCurdy, D., Strigo, I., Matthews, S., Yang, T., Irvine, Z., & Kaye, W. (2013). Greater anterior insula activation during anticipation of food images in women recovered from anorexia nervosa versus controls. *Psychiatry Research - Neuroimaging*, *214*(2), 132–141. https://doi.org/10.1016/j.pscychresns.2013.06.010
88. Oomen, C. A., Soeters, H., Audureau, N., Vermunt, L., van Hasselt, F. N., Manders, E. M. M., Joëls, M., Lucassen, P. J., & Krugers, H. (2010). Severe early life stress hampers spatial learning and neurogenesis, but improves hippocampal synaptic plasticity and emotional learning under high-stress conditions in adulthood. Journal of Neuroscience, 30(19), 6635–6645. https://doi.org/10.1523/JNEUROSCI.0247-10.2010
89. Parb, W. P., Vincent, G. P., Isom, K. E., & Reeves, J. M. (1978). sex differences a n d incidence of activity-stress ulcers in the rat'. In *Psychological Reports* (Vol. 43).
90. Paternain, L., Martisova, E., Milagro, F. I., Ramírez, M. J., Martínez, J. A., & Campión, J. (2012). Postnatal maternal separation modifies the response to an obesogenic diet in adulthood in rats. *DMM Disease Models and Mechanisms*, *5*(5), 691–697. https://doi.org/10.1242/dmm.009043
91. Paulukat, L., Frintrop, L., Liesbrock, J., Heussen, N., Johann, S., Exner, C., Kas, M. J., Tolba, R., Neulen, J., Konrad, K., Herpertz-Dahlmann, B., Beyer, C., & Seitz, J. (2016). Memory impairment is associated with the

loss of regular oestrous cycle and plasma oestradiol levels in an activity-based anorexia animal model. *The world journal of biological psychiatry : the official journal of the World Federation of Societies of Biological Psychiatry*, *17*(4), 274–284. https://doi.org/10.3109/15622975.2016.1173725

92. Pryce, C. R., & Feldon, J. (2003). Long-term neurobehavioural impact of the postnatal environment in rats: Manipulations, effects and mediating mechanisms. *Neuroscience and Biobehavioral Reviews*, *27*(1–2), 57–71. https://doi.org/10.1016/S0149-7634(03)00009-5
93. Quaedflieg, C. W. E. M., Schwabe, L., Meyer, T., & Smeets, T. (2013). Time dependent effects of stress prior to encoding on event-related potentials and 24h delayed retrieval. *Psychoneuroendocrinology*, *38*(12), 3057–3069. https://doi.org/10.1016/j.psyneuen.2013.09.002
94. Ratnovsky Yevgeniya, Y., & Neuman, P. (2011). The effect of pre-exposure and recovery type on activity-based anorexia in rats. *Appetite*, *56*(3), 567–576. https://doi.org/10.1016/j.appet.2011.01.027
95. Rees, S. L., & Fleming, A. S. (2001). How early maternal separation and juvenile experience with pups affect maternal behavior and emotionality in adult postpartum rats. *Animal Learning & Behavior, 29*(3), 221–233. https://doi.org/10.3758/BF03192889
96. Ribasés, M., Gratacòs, M., Armengol, L., de Cid, R., Badía, A., Jiménez, L., Solano, R., Vallejo, J., Fernández, F., & Estivill, X. (2003). Met66 in the brain-derived neurotrophic factor (BDNF) precursor is associated with anorexia nervosa restrictive type. *Molecular Psychiatry*, *8*(8), 745–751. https://doi.org/10.1038/sj.mp.4001281
97. Rorty, M., Yager, J., & Rossotto, E. (1994). Childhood sexual, physical, and psychological abuse in bulimia nervosa. *American Journal of Psychiatry*, *151*(8), 1122–1126. https://doi.org/10.1176/ajp.151.8.1122
98. Rosenfeld, C. S. (2017). Sex-dependent differences in voluntary physical activity. In Journal of Neuroscience Research (Vol. 95, Issues 1–2, pp. 279–290). John Wiley and Sons Inc. https://doi.org/10.1002/jnr.23896
99. Routtenberg, A., & Kuznesof, A. W. (1967). Self-starvation of rats living in activity wheels on a restricted feeding schedule 1. In Journal of Comparative and Physiological Psychology (Vol. 64, Issue 3).
100. Sakurada, S., Shido, O., Sugimoto, N., Hiratsuka, Y., Yoda, T., & Kanosue, K. (2000). Autonomic and behavioural thermoregulation in starved rats. *The Journal of physiology*, *526 Pt 2*(Pt 2), 417–424. https://doi.org/10.1111/j.1469-7793.2000.00417.x
101. Sapolsky', R. M., & Meaney', M. J. (1986). Maturation of the Adrenocortical Stress Response: Neuroendocrine Control Mechanisms and the Stress Hyporesponsive Period. In *Brain Research Reviews* (Vol. 11).
102. Schalla, M. A., & Stengel, A. (2019). Activity based anorexia as an animal model for anorexia nervosa–a systematic review. In *Frontiers in Nutrition* (Vol. 6). Frontiers Media S.A. https://doi.org/10.3389/fnut.2019.00069
103. Schorr, M., & Miller, K. K. (2017). The endocrine manifestations of anorexia nervosa: Mechanisms and management. In *Nature Reviews Endocrinology* (Vol. 13, Issue 3, pp. 174–186). Nature Publishing Group. https://doi.org/10.1038/nrendo.2016.175

104. Schultz, W. (1998). *Predictive Reward Signal of Dopamine Neurons. Journal of Neurophysiology, 80(1), 1–27.* doi:10.1152/jn.1998.80.1.1

105. Schultz, W. (2010). *Open Access REVIEW BioMed Central Dopamine signals for reward value and risk: basic and recent data*. http://www.behavioralandbrainfunctions.com/content/6/1/24

106. Seibenhener, M. L., & Wooten, M. C. (2015). Use of the open field maze to measure locomotor and anxiety-like behavior in mice. Journal of Visualized Experiments, 96. https://doi.org/10.3791/52434

107. Skunde, M., Walther, S., Simon, J. J., Wu, M., Bendszus, M., Herzog, W., & Friederich, H. C. (2016). Neural signature of behavioural inhibition in women with bulimia nervosa. *Journal of Psychiatry and Neuroscience*, *41*(5), E69–E78. https://doi.org/10.1503/jpn.150335.https://doi.org/10.1016/j.neubiorev.2015.11.003

108. Södersten, P., Bergh, C., Leon, M., & Zandian, M. (2016). Dopamine and anorexia nervosa. In Neuroscience and Biobehavioral Reviews (Vol. 60, pp. 26–30). Elsevier Ltd. https://doi.org/10.1016/j.neubiorev.2015.11.003

109. Szczypka, M. S., Kwok, K., Brot, M. D., Marck, B. T., Matsumoto, A. M., Donahue, B. A., & Palmiter, R. D. (2001). Dopamine production in the caudate putamen restores feeding in dopamine-deficient mice. *Neuron*, *30*(3), 819–828. https://doi.org/10.1016/s0896-6273(01)00319-1

110. Tjong, Y. W., Po Ip, S., Lao, L., Wu, J., Fong, H. H. S., Sung, J. J. Y., Berman, B., Che, C. T., & Ip, S. P. (2010). Neonatal maternal separation elevates thalamic corticotrophin releasing factor type 1 receptor expression response to colonic distension in rat. In *Neuroendocrinol Lett* (Vol. 31, Issue 2). www.nel.edu

111. Tritsch, N. X., Oh, W. J., Gu, C., & Sabatini, B. L. (2014). Midbrain dopamine neurons sustain inhibitory transmission using plasma membrane uptake of GABA, not synthesis. *ELife*, *2014*(3). https://doi.org/10.7554/eLife.01936

112. van Elburg, A. A., Kas, M. J. H., Hillebrand, J. J. G., Eijkemans, R. J. C., & van Engeland, H. (2007). The impact of hyperactivity and leptin on recovery from anorexia nervosa. *Journal of Neural Transmission*, *114*(9), 1233–1237. https://doi.org/10.1007/s00702-007-0740-6

113. van Kuyck, K., Casteels, C., Vermaelen, P., Bormans, G., Nuttin, B., & Van Laere, K. (2007). Motor- and food-related metabolic cerebral changes in the activity-based rat model for anorexia nervosa: a voxel-based microPET study. *NeuroImage*, *35*(1), 214–221. https://doi.org/10.1016/j.neuroimage.2006.12.009

114. van Leeuwen, S. D., Bonne, O. B., And, A., Berry, E. M., Leeuwen, V., Bonne, O. B., Avraham, Y., & Berry, E. M. (1997). Separation as a New Animal Model for Self-Induced Weight Loss. In *Physiology & Behavior* (Vol. 2659, Issue 6).

115. Varga, J., Ferenczi, S., Kovács, K. J., Garafova, A., Jezova, D., & Zelena, D. (2013). Comparison of Stress-Induced Changes in Adults and Pups: Is Aldosterone the Main Adrenocortical Stress Hormone during the Perinatal Period in Rats? *PLoS ONE*, *8*(9). https://doi.org/10.1371/journal.pone.0072313

116. Varga, J., Fodor, A., Klausz, B., & Zelena, D. (2015). Anxiogenic role of vasopressin during the early postnatal period: Maternal separation-induced ultrasound vocalization in vasopressin-deficient Brattleboro rats. Amino Acids, 47(11), 2409–2418. https://doi.org/10.1007/s00726-015-2034-x

117. Verhagen, L. A. W., Luijendijk, M. C. M., Hillebrand, J. J. G., & Adan, R. A. H. (2009). Dopamine antagonism inhibits anorectic behavior in an animal model for anorexia nervosa. *European Neuropsychopharmacology*, *19*(3), 153–160. https://doi.org/10.1016/j.euroneuro.2008.09.005

118. Walf, A. A., & Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nature Protocols, 2(2), 322–328. https://doi.org/10.1038/nprot.2007.44

119. Walsh, R. N., Cummins, R. A. (1976) The open field test: a critical review. Psychol. Bull. 83, 482-504

120. Welch, A. C., Katzka, W. R., & Dulawa, S. C. (2018). Assessing activity-based anorexia in mice. *Journal of Visualized Experiments*, *2018*(135). https://doi.org/10.3791/57395

121. Wu, H., van Kuyck, K., Tambuyzer, T., Luyten, L., Aerts, J. M., & Nuttin, B. (2014). Rethinking food anticipatory activity in the activity-based anorexia rat model. Scientific Reports, 4. https://doi.org/10.1038/srep03929

122. Yamamuro, Y., & Sensui, N. (2000). Maternal Behavior and Emotional Status of Mother Rats at Different Stages of Lactation. In *Animal Science Journal* (Vol. 71, Issue 2).

123. Zapata, R. C., Singh, A., Ajdari, N. M., & Chelikani, P. K. (2018). Dietary Tryptophan Restriction Dose-Dependently Modulates Energy Balance, Gut Hormones, and Microbiota in Obesity-Prone Rats. Obesity, 26(4), 730–739. https://doi.org/10.1002/oby.22136

124. Zgheib, S., Méquinion, M., Lucas, S., Leterme, D., Ghali, O., Tolle, V., Zizzari, P., Bellefontaine, N., Legroux-Gérot, I., Hardouin, P., Broux, O., Viltart, O., & Chauveau, C. (2014). Long-Term physiological alterations and recovery in a mouse model of separation associated with time-restricted feeding: A tool to study anorexia nervosa related consequences. *PLoS ONE*, *9*(8). https://doi.org/10.1371/journal.pone.0103775

125. Zhou, Q.-Y., & Palmiter, R. D. (1995). Dopamine-Deficient Mice Are Severely Hypoactive, Adipsic, and Aphagic. In *Cell* (Vol. 83).